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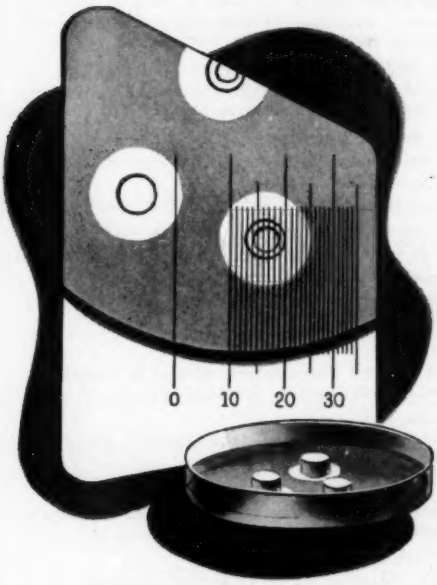
Penicillin



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A Study of Normal Bovine Serum Solids with Vitamin K Added as an Oral Prophylactic for Calf Scours I. Use of Healthy Newborn Calves

G. W. ANDERSON, D.V.M., M.S.; W. M. DuPRE, B.S.; J. P. LaMASTER, M.S.

Clemson, South Carolina

THE PRESENCE of scours in calves of all ages is a major hazard in the development of replacement stock in the dairy and beef cattle industries. Many dairy farmers have ceased raising calves because of the high mortality from scours. There are many different types and causes of calf scours. Bacterial white scours, herein after referred to as primary scours, occurs in the newborn calf. Bacterial, coccidial, verminous, and nutritional scours occur in calves 2 weeks to 10 months old. The symptoms of these diseases are loss of weight and general unthriftiness. Death occurs in many cases. When the animal lives, there is a slow gain in weight and growth.

This paper is concerned with the bacterial white scours (primary scours) of the newborn calf.

EARLY STUDIES

One of the earliest studies of scours was that of Smith and Little.[15] Nine control calves which did not receive colostrum died; all receiving colostrum survived. Ragsdale and Brody[16] fed whole milk to calves receiving no colostrum and reported a mortality rate of 32 per cent. Nelson[12] reported: "Normal blood serum of the dairy cow regularly contains anti-coli agglutinins. Anti-coli agglutinins are not present in the blood serum of the new-born calf. Anti-coli agglutinins must be transmitted from the cow to the calf by way of the colostrum."

BACTERIAL AND NUTRITIONAL STUDIES

Many investigators[1-25] studied the calf scour complex with reference to cause and treatment. An analysis of the available research indicated that practically all calf scours, excluding internal parasites and coccidia, were bacterial in nature and that the method of treatment was bactericidal. Other investigators believed it to be dietary and that the treatment should be nutritional.

Vitamin therapy has been used with some success. The vitamins most commonly used are vitamins A, B, riboflavin, pantothenic acid, choline, and nicotinic acid.[18] As soon as a new vitamin has been discovered, it has been used, but at times with negligible results.

The bactericidal treatment of calf scours has been somewhat successful, especially when begun during the initial phases of the disease. Sulfonamide prophylaxis has also proved satisfactory in some problem herds. The compounds most commonly used in treatment and prophylaxis are sulfaguanidine, sulfamerazine, sulfathalidine, sulfasuccidine, sulfathiazole, and sulfamethazine.

TABLE I—Composition of Three Preparations Used in Studying the Control of Primary Scours

Preparation	Serum solids	Modified milk solids	Vitamin K
31	100 Gm.	.....	37.5 mg.
32*	.....	100 Gm.	37.5 mg.
33	100 Gm.	100 Gm.	37.5 mg.

*Preparation 32 served as a control.

Wise[26] and Wise and Anderson[27, 28, 29] investigated primary scours in the Clemson College dairy herd and found sulfaguanidine and sulfathalidine to be satisfactory compounds for

From the Dairy Department, South Carolina Experiment Station, Clemson College, Clemson. Associate animal pathologist (Anderson), assistant dairy husbandman (DuPre), and head of the Dairy Department (LaMaster).

Technical publication No. 193, presented with the approval of the director of the South Carolina Agricultural Experiment Station.

The authors are indebted to Dr. Chris P. Segard, Wisconsin Alumni Research Foundation, New York, N. Y., and Dr. John W. Palmer, E. R. Squibb and Sons, New Brunswick, N. J., for their assistance.

TABLE 2—The Results Obtained from the Use of Preparation 31 (Serum Solids and Vitamin K) on Group 1

Calf (No.)	Born	Feedings of preparation 1st	2nd	3rd	Results
G-483	5 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Scoured third day, normal fourth day
BX-109	6 p.m.	6 a. m.—50 Gm.	4 p.m.—50 Gm.	.....	Very loose stools second and third days, normal fourth day
C-481	12 noon	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
GB-357	3 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
A-407	5 a. m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
A-409	9 a. m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Slightly loose stool second day, normal third day
GB-362	9 a. m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Pasty stool second day, otherwise normal
GB-364	2 p. m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Slightly loose stool second and third day, normal fifth day
C-472	6 a.m.	9 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Very loose stool second day, normal fourth day
BX-108	5 a.m.	9 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Very loose stools second and third days, normal fourth day
C-476	4 a.m.	8 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Pasty stool second day, normal third day
C-471	11 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	Normal
GB-354	5 a.m.	7 a.m.—50 Gm.	2 p.m.—50 Gm.	5 a.m.—50 Gm.	Very loose and watery stool second day, rec'd 900,000 units of penicillin and 40 cc. of sulmet third day, normal fourth day
A-404	10 a.m.	6 p.m.—50 Gm.	6 a.m.—50 Gm.	6 p.m.—50 Gm.	Emaciated, scoured second and third days, normal fifth day
BX-105	10 a.m.	6 p.m.—50 Gm.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	Normal
BX-112	5 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
C-488	4 p.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Very loose stools third and fourth days, loose fifth day, normal sixth day
B-58	2 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Bloody, pasty stool second day, normal third day
C-492	9 p.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
GB-368	2 a.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal

TABLE 3—The Results Obtained from the Use of Preparation 32 (Modified Milk Solids and Vitamin K) on Group 2 (Controls)

Calf (No.)	Born	Feedings of preparation 1st	2nd	3rd	Results
C-470	11 a.m.	3 p.m.—50 Gm.	9 p.m.—50 Gm.	7 a.m.—50 Gm.	Normal
BX-106	4 a.m.	7 a.m.—50 Gm.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	Normal
G-407	6 a.m.	8 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Sick second day, rec'd sulmet and penicillin, (not scours, died 2 p.m. second day)
C-473	9 p.m.	7 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal
C-475	3 a.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal
GB-355	4 a.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Pasty stool second day, normal third day
C-477	6 a.m.	8 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Loose stool second day, normal third day
A-405	9 p.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal
C-480	3 a.m.	7 a.m.—50 Gm.	6 p.m.—50 Gm.	.....	Normal
BX-110	11 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
GB-358	4 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
A-408	5 p.m.	9 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Loose stool third day, normal fourth day
GB-360	2 p.m.	6 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
GB-363	5 a.m.	10 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal
A-411	?	5 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Loose stools second to fourth days, normal sixth day
BX-113	12 noon	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Loose stools second day, normal fourth day
BX-114	8 p.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Loose stools third day, normal fourth day
GB-366	9 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
C 493	2 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Normal
A-412	4 p.m.	6 a.m.—50 Gm.	4 p.m.—50 Gm.	.....	Normal
C-495	11 a.m.	4 p.m.—50 Gm.	6 a.m.—50 Gm.	.....	Loose stools third day, normal fourth day

prophylaxis and treatment. They also found that prophylaxis was more satisfactory than the treatment of a sick calf. However, about 3 per cent mortality, apparently due to kidney blockage, occurred in well calves.

The symptoms of primary scours are loss of appetite, listlessness, scours followed by salivation, dehydration, and death in forty-eight to 120 hours after birth. Lesions are confined to the gastrointestinal tract and are present as edema and hyperemia of the abomasum. Edema and petechial and confluent hemorrhages also occur in the mucous membrane of the intestines. *Escherichia coli* is the organism most commonly isolated.

The severe dehydration caused by the loss of fluid through salivation and scouring, as shown by (among other things) the sunken eyes and general appearance, is considered the cause of death. The stagnation of the blood produces anoxemia and suffocation. The drying of the body cells and tissues slows up metabolism with its accumulation of body poisons.

EARLY THERAPY

One of the earliest therapeutic methods used in the treatment of calf scours was the intravenous injection of the dam's blood into the sick calf, the idea being that the antibodies in the dam's blood would give a passive immunity until the calf could develop an active immunity. The major disadvantages of this method were the difficult administration into a flaccid vein and the inability of the approximately 2-qt. capacity of the blood vascular system of the calf to accept the large quantities of dam's blood necessary to effect a cure.

As the blood of the newborn calf contains very few antibodies, they must ordinarily be derived from the dam's colostrum. A passive immunity occurs in the calf as soon as the immune protein molecules pass through the mucous membrane of the intestine into the blood stream. If insufficient immunity is produced, the calf may develop primary scours. The mucous membrane is apparently pervious to the immune protein molecules until about twenty-four to thirty hours after birth.

The oral administration of dehydrated bovine serum solids during the period of permeability of the mucous membrane should increase greatly the amount of antibodies in the calf's blood and prevent the development of primary scours.

This method of therapy prompted a study of normal bovine serum solids with vitamin K in a water suspension administered *per os* to healthy bull calves. Vitamin K was added to control the hemorrhage that often occurs in primary scours.*

EXPERIMENTAL

Preliminary research was conducted with healthy bull calves. Some of the calves remained with the dams for four days after birth; other calves were transferred at birth to individual pens. The calves were divided into three groups and given, respectively, preparations 31, 32, and 33.** The composition of the preparations is given in table 1.

The serum solids were prepared from normal healthy beef cattle blood collected at slaughterhouses. The blood was defibrinated, centrifuged to remove red blood cells, filtered through bacteria-excluding filters, and lyophilized. Bacteriological tests were conducted with guinea

*Karl Paul Link, University of Wisconsin, unpublished data.

**Prepared and furnished by E. R. Squibb & Sons, under the name plasmylac.

TABLE 4—The Results Obtained from the Use of Preparation 33 (Equal Parts of Serum Solids and Modified Milk Solids with Vitamin K) on Group 3

Calf (No.)	Born	Feedings of preparation			Results
		1st	2nd	3rd	
G-406	11 a.m.	3 p.m.—100 Gm.	9 p.m.—100 Gm.	7 a.m.—100 Gm.	Calf lazy and breathing fast second day, stools normal throughout
H-613	5 a.m.	7 a.m.—100 Gm.	4 p.m.—100 Gm.	6 a.m.—100 Gm.	Normal
BX-107	1 p.m.	5 p.m.—100 Gm.	6 a.m.—100 Gm.	4 p.m.—100 Gm.	Loose stool third day, normal at all other times
C-474	4 a.m.	8 a.m.—100 Gm.	4 p.m.—100 Gm.	.....	Normal
GB-356	2 p.m.	6 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Loose stools second day, normal third day
C-478	5 p.m.	5 a.m.— 50 Gm.	4 p.m.— 50 Gm.	.....	Normal
C-479	4 p.m.	5 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Normal
C-482	8 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Normal
A-406	3 a.m.	9 a.m.— 50 Gm.	4 p.m.— 50 Gm.	.....	Normal
GB-359	3 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Normal
BX-111	5 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Slightly loose stool second day, normal third day
C-486	9 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Normal
C-487	11 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Loose stools second day, normal fourth day
GB-365	10 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Loose stools third day, normal fourth day
C-491	6 a.m.	4 p.m.— 50 Gm.	6 a.m.— 50 Gm.	.....	Normal
C-494	9 p.m.	9 a.m.— 50 Gm.	4 p.m.— 50 Gm.	.....	Normal

pigs to be certain that the serum solids were free of infectious agents. The milk solids were spray-dried enzylac milk, a milk modified by partial enzymatic digestion. Vitamin K was added in the form of menadione.

Group 1, composed of 20 calves, received preparation 31 (serum solids and vitamin K). Table 2 shows the results obtained. Group 2, the control group, was composed of 21 calves and received preparation 32 (modified milk solids and vitamin K). Table 3 shows the results obtained from the use of this compound. Group 3, composed of 16 calves, received preparation 33 (equal parts of serum solids and modified milk solids with vitamin K). Table 4 shows the results obtained from the use of this preparation.

DISCUSSION

Loose stools were observed in 4 calves receiving 50-Gm. doses of preparation 31 (serum solids and vitamin K), suspended in water. These stools were thinner in consistency than those occurring in groups 2 and 3. This condition may be alleviated by the addition of a small quantity of milk solids to the serum solids.

Calves GB-354 and A-404 (table 2) apparently developed primary scours, and an additional 50 Gm. of preparation 31 was given to them. Calf GB-354 showed symptoms of pneumonia and received 900,000 units of penicillin. These calves recovered completely.

Calves C-471 and BX-105 (table 2) were given an additional 50 Gm. of preparation 31 and remained normal.

Six calves (table 3), receiving 50-Gm., water-suspended doses of preparation 32 (modified milk solids and vitamin K), had loose stools, probably a very mild form of primary scours. Calf G-407 died of an infection other than primary scours.

Preparation 33 (serum solids, modified milk solids, and vitamin K) was administered in 100-Gm., water-suspended doses to 4 calves (table 4). Three of the 4 calves received an additional 100-Gm. treatment. Since the 100-Gm. quantity was difficult to administer, subsequent feedings given to 12 calves were reduced to 50 Gm. each. Four calves in this group developed loose stools but became normal seventy-two to ninety-six hours later. These loose stools may have been a mild form of primary scours.

CONCLUSIONS

1) Normal bovine serum solids with vitamin K were not harmful to the healthy newborn calf.

2) Two calves in group 1, which received serum solids and vitamin K only, apparently developed primary scours. They recovered, probably because of the administration of more of the preparation. The loose stools in groups 2 and 3 were thought to be a mild form of primary scours.

3) The addition of modified milk solids to the serum solids-vitamin K preparation appeared to reduce the gastrointestinal upset which occurs in some normal calves.

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The Comparative Effect of Related Chemical Groups on the Growth of Three Species of Brucella

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SINCE HUDDLESON,[3] in 1931, recommended using the bacteriostatic action of dyes as a means of differentiating the three species of Brucella, little has been reported to elucidate the mechanism involved in this phenomenon. On the assumption that if the mechanism were known it would provide information on the metabolic activity of the three species of Brucella, and since attempts to formulate synthetic mediums capable of supporting the growth of Brucella through continuous subculture have not been entirely satisfactory, it was decided to test the bacteriostatic action of various chemical groups in the hope that the structure of these related compounds might provide a clue to the factors influencing the growth of each species.

Because of its initial use in classifying the species, and since a standard classification was available, the compounds selected were included in three of the major dye groups. The classification followed was that used by the Commission on Standardization of Biological Stains,[2] a system of classification based upon the chromophore present in the compounds. This system essentially affords a grouping of the dyes according to their general chemical structure.

MATERIALS AND METHODS

A preliminary screening was done in order to eliminate dyes that would be of no value in a survey of this nature. Those that proved to be insoluble in either distilled water or 95 per cent ethyl alcohol, would not withstand heat sterilization, or were nontoxic to the three species in a high concentration were not included in this study. For example, in the case of fast green in the phenyl methane group, growth was not inhibited at a concentration of 10 mg. per cubic centimeter of medium.

The cultures of Brucella used were obtained from various laboratories and had been differentiated as *Brucella suis*, *Brucella abortus*, or *Brucella melitensis*. Since all cultures of *Br. abortus* had been under cultivation for considerable time, increased carbon dioxide was not required and was not used at any time during this survey. One strain of *Br. abortus* (strain 19), two of *Br. suis*, and two of *Br. melitensis* were used in the investigations.

The basic medium employed was tryptose agar (Difco). The dyes were prepared in stock solutions of varying concentrations and sterilized if necessary (as determined by a plate test). The dye solutions were made as concentrated as possible and, except in isolated instances, it was not necessary to add more than 2.0 cc. of dye to 100 cc. of the medium. The desired amount was added to the melted tryptose agar, thoroughly mixed, and the plates poured. In the results, the concentration of dye in the medium is therefore expressed in terms of weight of dye per cubic centimeter of medium rather than as a dilution.

Plate inoculations were made with a standard loop from a saline suspension of the particular species. First readings of the plates were made after forty-eight hours' incubation but, although the plates were not discarded for five days, holding them for longer than seventy-two hours did not materially affect the results. Each test was controlled on tryptose agar. The results from each group are summarized in table 1. In group 1 (the quinoneimide dyes), thionine, as expected, inhibited quantitatively *Br. abortus* and *Br. melitensis*. *Brucella suis* grew luxuriantly at concentrations causing complete inhibition of both *Br. abortus* and *Br. melitensis*. However, thionine proved to be the only dye in the group with this specific characteristic. The remaining dyes of this group had an entirely different quantitative action in that both *Br. abortus* and *Br. suis* were inhibited at concentrations favorable to *Br. melitensis*. Compounds of this group that favored the growth of *Br. melitensis* over the other two species were Capri blue, Meldola's blue, and Safranin O. The latter compound proved the superior of all others in the group in differentiating *Br. melitensis* from the other two species. Excellent growth was apparent at 300 μg. per cubic centimeter and was not inhibited until a concentration of 600 μg. per cubic centimeter was reached. *Brucella abortus* and *Br. suis* both grew scantily, even on very low concentrations.

The phenyl methane group contained basic fuchsin, which like thionine was one of the

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standard dyes used in differentiating the species of Brucella. Specifically, it permitted the growth of *Br. abortus* and *Br. melitensis* at concentrations inhibiting *Br. suis*. Also, there was a significant quantitative difference in the tolerance of this compound by *Br. abortus* and *Br. melitensis*. On the other hand, the xanthenes showed a more definite action in the presence of Brucella. There was a constant marked difference in toleration by the various species. *Brucella melitensis* was, with the exception of pyronin, inhibited at much lower concentrations than the other two species.

DISCUSSION

The three major chemical groups surveyed in this study are related structurally since they all contain the quinoid-benzene rings as the chromophore. Further close chemical relationship exists between the xanthene and triphenyl methane groups, both being derived from the same parent hydrocarbon, triphenyl methane.[1] The xanthene group therefore is usually included in the triphenyl methane group as a more complex derivative of the parent substance rather than as a separate entity. This actually leaves two separate chemical groups related to each other by a common chromophore. If the basic structure *per se* of the compound is intimately involved in the effect upon the growth of the three species of Brucella, one would anticipate a definite pattern of action from these two groups, changing as the derivatives become more complex.

The quinone-imide group as a whole does not give a consistent type of reaction. Even with two chemicals that are as structurally similar as thionine and methylene blue (tetramethyl thionine), there is no similarity in their action on the three species of Brucella. The only general pattern of action that may be found in this group as a whole is that, with the exception of thionine, all are more toxic to *Br. abortus* and *Br. suis* than they are to *Br. melitensis*.

The triphenyl methane group exclusive of the xanthenes is also less toxic for *Br. melitensis* than for the other two species, but again there is no consistency in the mode of action of the group as a whole. With the xanthenes having the identical chromophore and theoretically coming from

TABLE I—The Bacteriostatic Effect of Various Chemical Groups on the Genus Brucella

	Group and name of dye	Concentration causing bacteriostasis					
		Br. abortus		*Br. suis*		*Br. melitensis*	
1	Quinone-imide group						
	Thionine	19.00	µg./cc.	100.00	µg./cc.	18.00	µg./cc.
	Methylene blue	1.00	µg./cc.	1.00	µg./cc.	5.00	µg./cc.
	Methylene azure	0.09	µg./cc.	0.09	µg./cc.	3.00	µg./cc.
	Methylene violet	1.00	µg./cc.	2.00	µg./cc.	4.00	µg./cc.
	Methylene green	7.00	µg./cc.	15.00	µg./cc.	21.00	µg./cc.
	Toluidine blue O	1.00	µg./cc.	2.00	µg./cc.	3.00	µg./cc.
	New methylene blue	1.00	µg./cc.	0.30	µg./cc.	3.00	µg./cc.
	Brilliant cresyl blue	3.00	µg./cc.	4.00	µg./cc.	6.00	µg./cc.
	Cresyl echt violet	2.00	µg./cc.	4.00	µg./cc.	40.00	µg./cc.
	Capri blue	1.00	µg./cc.	1.00	µg./cc.	20.00	µg./cc.
	Meldola's blue	35.00	µg./cc.	38.00	µg./cc.	100.00+	µg./cc.
	Celestian blue	80.00	µg./cc.	81.00	µg./cc.	80.00	µg./cc.
	Neutral red	....*	.....	100.00	µg./cc.	800.00	µg./cc.
	Safranin O	15.00	µg./cc.	1.00	µg./cc.	600.00	µg./cc.
	Amethyst violet	16.00	µg./cc.	13.00	µg./cc.	14.00	µg./cc.
2	Phenyl methane group						
	Auramin	21.00	µg./cc.	19.00	µg./cc.	25.00	µg./cc.
	Malachite green	2.00	µg./cc.	1.00	.....	10.00+	µg./cc
	Brilliant green	0.70	µg./cc.	1.40	µg./cc.	1.60	µg./cc.
	Basic fuchsin	10.00	µg./cc.	1.00	µg./cc.	30.00	µg./cc.
	Ethyl violet	80.00	µg./cc.	10.00	µg./cc.	120.00	µg./cc.
	Hoffman's violet	13.00	µg./cc.	4.00	µg./cc.	12.00	µg./cc.
	Crystal violet	6.00	µg./cc.	1.00	µg./cc.	7.00	µg./cc.
	Ethyl green	200.00	µg./cc.	140.00	µg./cc.	170.00	µg./cc.
	Coralline red	90.00	µg./cc.	40.00	µg./cc.	40.00	µg./cc.
	Victoria blue 4R	170.00	µg./cc.	.....*	µg./cc.	140.00	µg./cc.
	Victoria blue R	500.00	µg./cc.	500.00	µg./cc.	1000.00	µg./cc.
3	Xanthene group						
	Pyronin	1.00	µg./cc.	0.60	µg./cc.	0.80	µg./cc.
	Rhodamine B	1000.00+	µg./cc.	500.00	µg./cc.	400.00	µg./cc.
	Fluoroscien	2.00	mg./cc.	10.00	mg./cc.	1.00	mg./cc.*
	Eosin Y	800.00	µg./cc.	1000.00	µg./cc.	250.00	µg./cc.
	Ethyl eosin	400.00	µg./cc.	300.00	µg./cc.	100.00	µg./cc.
	Eosin B	900.00	µg./cc.	1000.00	µg./cc.	500.00	µg./cc.
	Erythrosin Y	1000.00+	µg./cc.	1000.00	µg./cc.	300.00	µg./cc.
	Phloxine B	500.00	µg./cc.	400.00	µg./cc.	90.00	µg./cc.
	Rose bengal	300.00	µg./cc.	600.00	µg./cc.	90.00	µg./cc.

*Lack of experimental data.

the parent hydrocarbon of the triphenyl methanes, this action is not now explainable.

SUMMARY AND CONCLUSIONS

Three of the major synthetic dye groups that are structurally related were tested for their effect upon the growth of the three species of Brucella.

The only consistent pattern within the group was the ability of the xanthene compounds to inhibit *Brucella melitensis* at concentrations much lower than those permitting growth of *Brucella suis* and *Brucella abortus*.

The structure *per se* of the compound was not wholly responsible for the variation in growth.

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The Determination of the Sedimentation Constant of a Homogeneous Component Having the Characteristics of the Foot-and-Mouth Disease Virus

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USING A Svedberg ultracentrifuge, Janssen[1] reported that foot-and-mouth disease virus of swine lymph has a sedimentation constant (s_{20}) of 17 to 28 S. More recently, it has been observed by the author that the virus can be quantitatively precipitated from about 5 ml. of infectious guinea pig lymph by centrifugation at 22,000 g. for 2.5 hours in 2-cm. diameter tubes of an SS-2 Servall vacuum centrifuge.[2, 3] Janssen obtained similar results using both infectious guinea pig and bovine lymph.[4, 5]

In theory, particles with s_{20} values of 17 to 28 S will not be quantitatively sedimented under the mild conditions which, in practice, have removed the virus from solution. This indicates that either the virus particles do not sediment as individual entities or that the above mentioned sedimentation constant does not hold, at least for guinea pig virus. The present report shows that the latter postulate is to be preferred, since it has been found that the s_{20} value of foot-and-mouth disease virus (guinea pig type 0 virus) is very probably 137 S, a value which theoretically permits the virus to be quantitatively sedimented as individual particles under the conditions described above. Furthermore, Galloway and Elford[6, 7] have presented much evidence that the virus of foot-and-mouth disease is monodisperse. Schlesinger and Galloway[8] also found that the sedimentation constant of various types of foot-and-mouth disease virus was greater than 17 to 28 S—that it was between 40 and 90 S. They used a Sharples supercentrifuge with an agar-coated bowl and estimated the sedimentation constant from the infectivity of the supernatants after centrifuging small volumes of virus for different intervals. While convection currents in their arrangement were negligible and sedimentation was radial, the sedimentation constant inferred may be in error, due to the mixing of precipitate and supernatant which occurred during the removal of the latter from the tube. In addition, tenfold differences in infectivity were the least that could be satisfactorily measured.

The following experiment, which was designed to determine the s_{20} value of guinea pig virus in a Spinco analytical ultracentrifuge, has been performed twice with nearly identical results.

METHODS AND RESULTS

The virus was first purified and concentrated from guinea pig lymph by two cycles of differential centrifugation. The starting material (i.e., clarified viral lymph), the two supernatants, and the two precipitate suspensions from the high speed runs were then titrated for infectivity in guinea pigs and also examined for high molecular weight components in the analytical centrifuge. As a control on the latter analysis, normal guinea pig lymph and ultracentrifugal fractions corresponding to those obtained for the infectious lymph were similarly examined.

The experimental details are as follows: 22 ml. of vesicular lymph was harvested from the plantae of 100 guinea pigs which had been inoculated twenty-four hours earlier with lymph from the 22nd serial passage of guinea pig type O virus. This was diluted with one-third its volume of M/15 phosphate buffer of pH 7.6, stored at 4 C. for two hours, and the fibrin removed by centrifugation at 3,500 revolutions per minute (r.p.m.). The following day, the buffered lymph was further clarified at 12,600 r.p.m. (11,700 ave. g.) for ten minutes in a Spinco preparative C rotor and designated as clarified viral lymph. Twenty-four milliliters of this solution was centrifuged for three hours at 3 C. in two tubes of the preparative A rotor at 23,150 r.p.m. (31,700 ave. g.) and the medium brake applied. The supernatant liquids from the two tubes (first ultrasupernatant) were removed with a capillary pipette, placed in a small reservoir, and stored at 4 C. Each centrifuge tube was inverted and its walls rinsed

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with buffer applied from a wash bottle, care being taken to prevent the buffer from coming into contact with the brownish gelatinous precipitate which, in this particular case, formed a narrow band along the side of the tube. The walls of the tube opposite the precipitate were dried with filter paper, the tube righted and placed in an ice bath. Each precipitate was suspended in 4.5 ml. of M/15 phosphate buffer of pH 7.6. This was done by first adding 0.25 ml. of buffer to the precipitate and grinding the mixture with a ground glass pestle; 0.75-, 1.5-, and 2-ml. portions of buffer were then added, each portion being thoroughly mixed with the precipitate suspension by forceful pipetting before adding the next. The resultant suspension was finally centrifuged at 3,500 r.p.m. for thirty minutes and the supernatant liquid designated as the first ultraprecipitate solution. All but 0.2 ml. of this supernatant was centrifuged for three hours at 6 C. in one tube of the preparative A rotor at 27,690 r.p.m. (45,300 ave. g.) and the medium brake applied. The supernatant (second ultrasupernatant) was removed as before, and the precipitate was suspended in 2 ml. of buffer in a manner similar to that used in suspending the first ultraprecipitate, except that the successive volumes of buffer used were 0.25, 0.5, 0.5, and 0.75 ml. After clarification at 3,500 r.p.m., the suspension was designated as the second ultraprecipitate solution. That it contained only a trace of protein, as shown by the sulfosalicylic acid test, indicated that the normal protein components of the lymph had been effectively eliminated by the purification procedure. If no losses occurred during the manipulations, there was effected a ninefold concentration of the high molecular weight proteins present in the crude viral lymph.

TABLE 1—Titration in Guinea Pigs of the Clarified Viral Lymph and of the First and Second Ultrasupernatants and Ultraprecipitate Solutions Obtained from It

Infectious material	Animals infected/noninfected at 10^{-x} dilution*								lethal dose$_{50}$[10]
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Clarified viral lymph	...	...	4/0	4/0	4/0	3/1	0/4	1/3	$10^{-6.5}$
1st ultrasupernatant	4/0	4/0	4/0	4/0	3/1	2/2	...	...	$10^{-5.8}$
1st ultraprecipitate solution	...	4/0	4/0	4/0	4/0	2/2	0/4	0/4	$10^{-6.0}$
2nd ultrasupernatant	4/0	4/0	4/0	0/4	0/4	0/4	...	...	$10^{-5.5}$
2nd ultraprecipitate solution	...	4/0	4/0	4/0	3/1	3/1	0/4	0/4	$10^{-6.2}$

*All dilutions are based on crude viral lymph as unit concentration.

The two cycles of differential centrifugation were completed in ten hours, and the five solutions previously referred to were immediately titrated for infectiousness in guinea pigs, 4 animals being used for each of the serial tenfold dilutions (in M/15 phosphate buffer of pH 7.6), shown in table 1.

The table shows that the virus was incompletely sedimented during the first high speed run. This result seemed at variance with those of experiments carried out as previously mentioned in the SS-2 Servall centrifuge. However, in later analytical determinations, a component with an s_{20} value of 137 S was indicated to be the virus, and it was calculated that a component having this s_{20} might not be completely removed from the upper portion of

TABLE 2—Sedimentation Constants of the High Molecular Weight Components Present in Normal and Viral Guinea Pig Lymph and in the Centrifugal Fractions Derived from Them

Material	Thickness of cell* (mm.)	Ave. cent. force** (g.)	Ave. temp.¶ (C.)	s_{20} (S)
Viral lymph				
Clarified viral lymph	18	161,250	6.3	18.5
1st ultrasupernatant	24	157,000	5.6	19.0
1st ultraprecipitate solution	4	.....	...	†
2nd ultrasupernatant	24	152,750	12.1	‡
2nd ultraprecipitate solution	30	92,500	9.2	137.0
Normal lymph				
Clarified normal lymph	24	161,500	13.5	19.0
1st ultrasupernatant	30	157,000	8.5	17.0
1st ultraprecipitate solution	..	.....	...	†
2nd ultrasupernatant solution	24	152,750	10.7	‡
2nd ultraprecipitate solution	30	.....	...	†

*All cells are sector shaped and of identical dimensions except for thickness. A 4-mm.-thick cell has a volume of approximately 0.25 ml. **The centrifugal force was first set at lower values and, if no peaks appeared within a reasonable period, the force was increased. ¶Average temperature in the cell during the photographic period and also in the viscosimeter. †No visible components. ‡No components with s_{20} greater than 7 S.

the clarified viral lymph when centrifuged in the preparative A tubes under the conditions of the experiment. The latter circumstance is in itself evidence for identifying the virus with the component having the s_{20} value of 137 S. Since the first ultraprecipitate had a large surface area, it is probable, however, that a considerable portion of the infectivity of the first ultrasupernatant was due to virus which had been peptized during the removal of the supernatants from the tubes. The centrifugal force in the second high speed run was higher than in the first, and the viscosity of the solution much less; the result being that the precipitate produced was in pellet form and contained nearly 99.9 per cent of the virus.

Following the preparative runs, a study was made in the analytical rotor of the protein

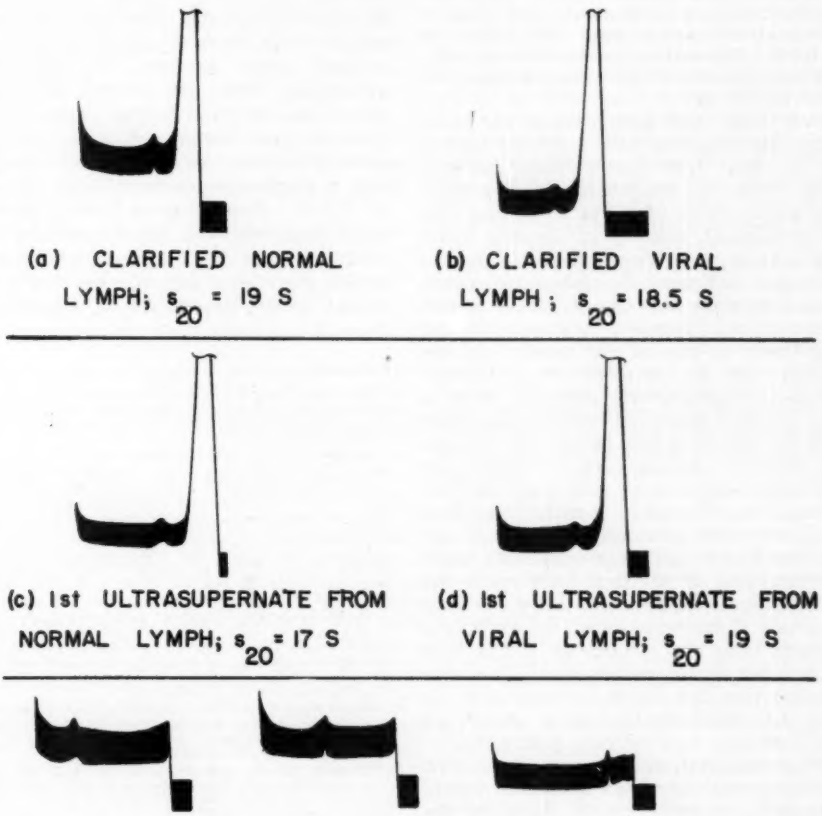
(a) CLARIFIED NORMAL LYMPH; s_{20} = 19 S
(b) CLARIFIED VIRAL LYMPH; s_{20} = 18.5 S
(c) 1st ULTRASUPERNATE FROM NORMAL LYMPH; s_{20} = 17 S
(d) 1st ULTRASUPERNATE FROM VIRAL LYMPH; s_{20} = 19 S
(e) 2nd ULTRAPRECIPITATE SOLUTION FROM VIRAL LYMPH; DIAGRAMS OF THE s_{20} - 137 S COMPONENT TAKEN AT EIGHT MINUTE INTERVALS. THE CORRESPONDING DIAGRAMS FOR NON-INFECTIOUS LYMPH SHOWED NO SEDIMENTING COMPONENTS

Fig. 1—Sedimentation diagrams of the viral solutions and controls. (Sedimentation is toward the left.)

components of the five solutions referred to previously and of corresponding control solutions prepared from normal guinea pig lymph. The normal lymph was obtained by directing a fine jet of steam onto the plantae of anesthetized animals and three hours later withdrawing the lymph from the vesicles which had formed. The sedimentation diagrams were made by the cylindrical lens method under the conditions given in table. 2. Included in the table are the s_{20} values for the high molecular weight protein components of the solutions. The density and viscosity values used in calculating the s_{20} were obtained by the use of a gradient tube and Ostwald viscosimeter, respectively. The partial specific volumes of the different components were conventionally assumed to be 0.749.[5, 9]

It is seen that both normal and viral guinea pig lymph contain a protein component in about equal concentration (as estimated from the sedimentation diagrams) with an s_{20} value of 17 to 19 S, and that this component remains in the first ultrasupernatants under conditions which almost invariably sediment the infectious agent. Thus, it appears that the 18.5 to 19 S component in viral lymph is a normal protein constituent of guinea pig lymph. In the purified and concentrated virus solution (second ultraprecipitate solution) there is only one high molecular weight component (137 S), and it is not present in the control solution. It has been calculated that this component will be quantitatively precipitated in a Servall SS-2 centrifuge under the conditions previously described, but may just fail to sediment completely under the conditions of the first high speed run of the present experiment. Since the sedimentation characteristics of the infectious agent fit these two conditions, and in view of the other considerations given, it is very probable that the 137 S component is the virus of foot-and-mouth disease (guinea pig type 0 virus).

The sedimentation diagrams of the viral solutions containing high molecular weight components, together with those of the controls, are shown in fig. 1. Significant stages in the sedimentation of the 137 S component are illustrated.

SUMMARY AND CONCLUSIONS

Guinea pig type 0 virus of foot-and-mouth disease was purified and concentrated by two cycles of differential centrifugation. Sedimentation diagrams of the clarified viral lymph and its ultracentrifugal fractions were made. Normal guinea pig lymph and fractions obtained from it by the same method as used with the viral lymph were similarly examined. It was found that both normal and viral lymph contained a protein component with a sedimentation constant (s_{20}) of 17 to 19 S. This protein was present in about equal concentration in both solutions and remained in the supernatants from the first high speed runs. The purified virus solution (ultraprecipitate suspension from the second differential run) was one-twelfth the volume of the clarified viral lymph and about six times more infectious ($12/10^{0.3}$), but contained only a single component with an s_{20} value of 137 S. From this and other evidence which is presented, it appears that the 17 to 19 S component is a normal constituent of guinea pig lymph, and that the 137 S component is very probably type 0 guinea pig virus of foot-and-mouth disease.

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An Investigation of the Foot-and-Mouth Disease Virus by the Use of Electrophoresis in Filter Paper

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RECENTLY A microelectrophoretic technique for the separation of proteins has been developed in which the protein molecules migrate on a strip of filter paper moistened with buffer solution. Turba and Enenkel,[1] Cremer and Tiselius,[2] and others have applied this method. Durrum[3] has fractionated human serum into its albumin, α_1-, α_2-, β-, and γ- globulin components on filter paper, and has also utilized the technique for the microionophoretic analysis of amino acid mixtures.

The purpose of the investigation reported here was to study the electrophoretic characteristics of the virus of foot-and-mouth disease (guinea pig strain O virus).

METHOD AND MATERIALS

The method of analysis was similar to that used by Durrum.[3] This consists simply of placing a drop of the protein solution at the center of a narrow strip of filter paper (0.01-0.001 ml. per cm. of paper width), the ends of which are dipping into electrode vessels containing buffer solution, moistening the paper with buffer solution as described later, and applying a source of direct current. After electrophoresis, the paper strip is removed from the apparatus, dried for five minutes at 100 C.,* and then immersed for five to ten minutes in 95 per cent alcohol which is saturated with mercuric chloride and which contains 1 Gm. of bromphenol blue per 100 ml. of solution. The strip is then washed in running water for ten minutes and again dried. Blue zones of the separated proteins are visible after this development, but the mobilities of the constituents can not be calculated because of the electroendosmosis which occurs concurrently with the electrophoretic separation.

The microelectrophoretic apparatus is illustrated in fig. 1 and is similar to that devised by Durrum. It is comprised of three rectangular glass containers (8 by 8 by 4 cm.), two of which serve as electrode vessels and one as a cover to retard evaporation from the paper strip. The electrode vessels carry carbon electrodes 1 cm. in diameter and are fitted with a plastic plate which is recessed to fit the glass vessels. This plate contains holes and slits through which the electrodes and filter paper fit and also carries a U-shaped glass rod over which the filter paper is placed.

In the present experiment, a Norwegian striated filter paper (H. Struers Chemiske Laboratorium, Copenhagen, Denmark) was used. The type of paper employed is very important. In this laboratory, it was found that a thick, hard paper gave a superior resolution of lymph protein components. Before the strip was placed in the apparatus, it was moistened with distilled water and dried at room temperature while draped over a glass rod of about 1 cm. diameter. This procedure gave preformed V-shaped strips with a smooth curvature at the apex and prevented the formation of sharp fractures at the apex, which occurred if the paper were bent before being moistened. Once the preformed strip had been placed in the position shown in fig. 1, a reference pencil mark was made at the apex and the protein solution applied from a capillary pipette. The buffer solution was then applied to the strip with a pipette at equal distances below the apex on both sides, permitting the buffer solution to be carried upward by capillarity to the protein position (apex). This procedure prevented the protein solution from running down the filter paper, as occurred if the protein were applied to a moistened filter paper strip. The cover vessel was put in place and, after about fifteen minutes, an electrical potential was applied across the carbon electrodes. The current was supplied from a well-filtered, full-wave rectifier through a potentiometer placed across the output terminals of the rectifier. By frequent adjustment of the potentiometer, the current through the paper was held constant.

Fresh infectious lymph was obtained from guinea pigs infected with the twenty-first serial passage of type 0 cattle virus in guinea pigs. It was taken from 24-hour-old primary vesicles and diluted with one part of veronal-acetate buffer** of pH 7.6 to four parts of lymph. (At this pH, phosphate buffer solution does not per-

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*This drying step can be omitted.

**Buffer composition: five volumes of a solution 0.143 M. in sodium acetate and 0.196 M. in sodium veronal, four volumes of 0.1 N. HCl, and 18 volumes of distilled water.

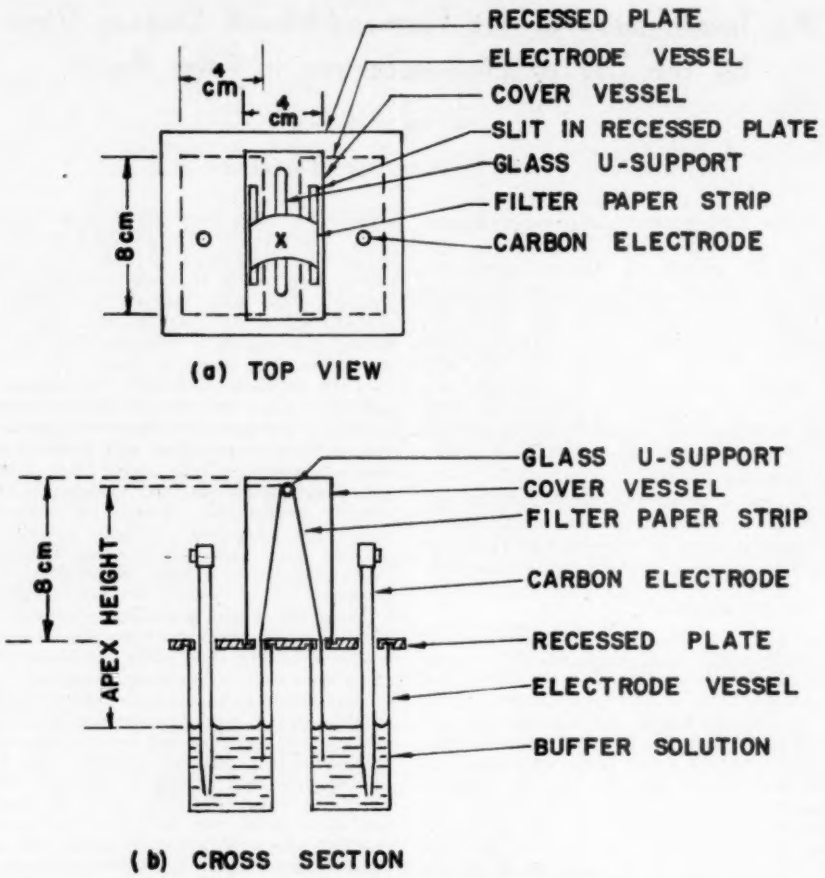

Fig. 1—Diagram of microelectrophoresis apparatus.

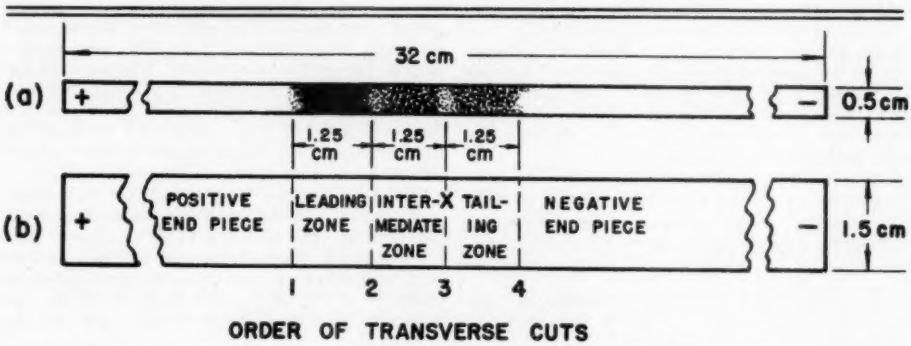

Fig. 2—(a) 0.5 by 32 cm. strip cut from original 2 by 32 cm. strip showing dyed protein zones. Cut from positive toward negative end.
(b) 1.5 by 32 cm. strip cut from original 2 by 32 cm. strip showing where cuts were made to obtain the five sections.

mit resolution of the protein mixture equivalent to that produced with the veronal-acetate system.) After standing two hours at 4 C., the crude buffered lymph was clarified by centrifugation at 3,500 revolutions per minute (r.p.m.).

EXPERIMENTAL AND RESULTS

Two-hundredths milliliter of the clarified lymph solution was applied to the apex (14.5 cm. above the level of the buffer solution) of a 2 by 32 cm. filter paper strip to form a narrow rectangular spot perpendicular to the long axis of the strip. The strip was then treated in the manner already described and a 2-ma. current (1 ma./cm. width) applied for two hours. The apparatus was maintained at 4 C. The original and final voltages necessary to maintain the 2-ma. current were 515 and 340 volts, respectively. The pH of the veronal-acetate buffer solution in the electrode vessels did not change appreciably under the conditions given.

After the current was cut off, the paper was removed from the apparatus and cut lengthwise into two strips, 0.5 and 1.5 cm. wide, respectively, starting at the positive end. The 1.5-cm. strip was supported over a wide beaker so that the apex remained upright, and the narrower strip was dyed without previous drying so that the electrophoretic pattern could be determined within five minutes after the current was stopped. Three blue protein zones were visible, each about 0.8 cm. wide. The leading zone, with the center at about 2 cm. toward the positive pole, consisted of about 70 per cent of the original lymph protein (estimated by color intensity). The other two zones, which were less deeply but equally dyed, had their centers at 0.7 and 0.4 cm. toward the positive and negative poles, respectively.

As indicated by the positions of the protein zones on the narrow strip, the 1.5-cm.-wide filter paper was cut into five sections. The sections obtained are shown in fig. 2 (b), the first cut being made between the positive end piece and leading zone section and so on toward the negative end of the paper.

The positive and negative end pieces were dyed by the standard procedure and gave no protein reaction. This indicates that these sections contained little or no protein, since the test detects as little as 28 μg. of serum protein when applied to filter paper in a 0.01-ml. volume.

The three middle sections (1.5 by 1.25 cm., each) were placed in individual micromortars and ground with glass fragments. The contents of each mortar were extracted three times with a total of 2 ml. of veronal-acetate buffer solution of pH 7.6 (0.67 ml. for each extraction) at 4 C. The paper pulp was centrifuged at 3,500 r.p.m., and the three supernatants and a sample of the original buffered lymph were titrated in 450-Gm. guinea pigs. The results of the titrations are shown in table 1. All dilutions were made in veronal-acetate buffer solution of pH 7.6 and are based on the concentration of crude unbuffered lymph. For this purpose, it was assumed that each of the three supernatants might contain a 0.005-ml. volume which was derived from the protein solution applied to the paper. Thus, while the infectivity of a zone can be compared with that of another zone, it can be compared with that of the original buffered lymph only if the above assumption, as to the distribution of liquid volumes, is nearly correct.

Table 1 shows that the ratio of the infectivities of the protein zones, starting with the leading zone, is 1:660:45. Thus, the leading zone, consisting mainly of albumin and perhaps some α-globulin,* contained an insignificant amount (ca. 0.1%) of the infectivity. The intermediate zone, consisting of α- and β- globulins,* contained about 93.5 per cent of the virus of foot-and-mouth disease (guinea pig O strain), and the tailing zone of γ- globulin about 6.4 per cent of the virus. It appears, therefore, that the virus studied has an electrophoretic mobility at pH 7.6 which is similar to that of the β- lymph

*That the relative positions of normal serum protein components obtained by filter paper electrophoresis is the same as those obtained in the Tiselius apparatus has been established by Durrum.[2]

TABLE 1—Infectivity of Guinea Pig Type O Lymph and of the Electrophoretic Zones Obtained with This Lymph by Electrophoresis in Filter Paper

Dilution of viral material*	Viral material			
	Buffered lymph	Supernatant, leading zone	Supernatant, intermediate zone	Supernatant, tailing zone
0.2×10^{-2}	4/0**	4/0	4/0	4/0
1.0×10^{-2}	4/0	0/4	4/0	4/0
10^{-4}	4/0	0/4	4/0	4/0
10^{-5}	4/0	0/4	4/0	1/3
10^{-6}	2/2	0/4	1/3	0/4
10^{-7}	0/4	0/4	0/4	0/4
10^{-8}	0/4	0/4	0/4	0/4
Lethal dose 50%	10^{-6}	0.14×10^{-2}	0.22×10^{-5}	0.32×10^{-4}

*See context for basis of dilutions. **Infected guinea pigs/noninfected guinea pigs.

globulins. This conclusion is in agreement with the work of Janssen,[4] who, using the Tiselius apparatus, found that the foot-and-mouth disease agent of cattle serum and lymph travels with a velocity near that of the a_2- and β- globulins.

SUMMARY

By the use of a method of microelectrophoresis in filter paper strips and only 0.02 ml. of guinea pig vesicle lymph diseased with foot-and-mouth disease virus (O strain), the velocity of the virus at pH 7.6 relative to that of normal lymph protein constituents was determined. More than 93 per cent of the infectivity of the virus was found in the middle zone of three distinct electrophoretic zones of protein. The leading, intermediate, and tailing zones contained about 0.1, 93.5 and 6.4 per cent, respectively, of the total infectivity, as determined by titration in guinea pigs. It is concluded that at pH 7.6 the viral particle has a mobility which is similar to that of the β- lymph globulins.

The small quantity of material necessary for the present analysis suggests that the method used may prove of great usefulness in orientation experiments on valuable protein solutions prior to further separation or quantitative analysis in the Tiselius-type apparatus.

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Research on Foot-and-Mouth Disease. Cultivation of the Foot-and-Mouth Disease Virus in Explanted Epithelium of the Bovine Tongue IV. Tissue and Medium Variations

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FOLLOWING THE successful propagation of the foot-and-mouth disease virus in bovine tongue epithelium,[1,2,3,5] a series of experiments was designed to test the effects of modifications of the tissues in the cultivating medium.

In searching for factors which might enhance the production of the virus, consideration was given to the role of the superficial layer of the bovine lingual epithelium, through which the virus probably penetrates to infect the deep layers with resulting vesicle formation. The logical approach to the problem seemed to be the use of the entire lingual epithelium, including the heavily contaminated, keratinous superficial layer, which had always been discarded in the cultivation process. To utilize the epithelium in this manner would simplify its harvest.

Since cysteine is present in keratinous proteins in relatively large amounts,[4] additional concentrations of cysteine were added to the mediums composed of Baker's fluid.[6] Similarly, to determine whether a catalytic substance was present in the keratinous superficial layer, extracts of this layer were added to the medium, in spite of the fact that Frenkel and van Waveren[1] did not observe, in their experiments, favorable action of aqueous extracts of tongue mucosa or extracts made with trypsin on the cultivation of foot-and-mouth virus in explanted bovine embryonic skin. The possibilities of using a medium other than Baker's fluid and the adaptation of the Mexican MP (Mexican Puebla) strain of Vallée type A virus[6] to artificial cultivation were also studied.

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METHODS AND RESULTS

Except for the variations that will be mentioned in each experiment, the methods of tissue harvest, composition of mediums, and other particulars of the virus cultivation were the same as reported in detail in a previous publication.[2]

The foot-and-mouth disease virus, Vallée type A, was propagated in minced bovine lingual epithelium in various fluid mediums, as indicated in table 1, and aërated with 95 per cent O_2 and 5 per cent CO_2. Contamination was controlled by the addition of penicillin and streptomycin. Incubation was of twenty-four hours' duration, and harvest was accomplished by grinding and centrifugating the culture tissue and filtering the supernatant fluid. The filtrate was buffered with a phosphate buffer of pH 7.4 and titrated in cattle by the use of the Henderson system[8] of ten intradermal, lingual sites per dilution and two dilutions per animal. Not more than 2 cattle were used per culture. We would have preferred to have made titrations on a greater number of cattle, but it was not feasible to do so at the time. The 50 per cent end point was determined by the method of Reed and Muench.[9]

Utilization of the entire epithelial layer of the bovine tongue was accomplished by first thoroughly scrubbing the tongue surface with a small, stiff, sterilized brush, liquid soap, and warm water. The scrubbing was followed by thorough rinsing and further cleansing with 70 per cent alcohol and cotton. The alcohol was allowed to evaporate and the tongues then were moistened with Fischer-Tyrode's solution, containing 2,500 units of penicillin and 12.5 mg. of streptomycin per 15 cc. of the solution. The superficial layer was next removed in the conventional manner by rolling off with a Frenkel roller,[2] and the deep epithelial layer which remained on the tongue was removed with a sterile razor blade. The entire epithelium, including the keratinous superficial layer, was minced with scissors. The entire tissue from one tongue was used to make two cultures, each containing 125 cc. of Baker's fluid and 15 cc. of seed virus.

While this increased the total amount of tissue for each culture from approximately

4 to 24 Gm., there was no increase of dividing epithelial cells. However, the number of cells that were susceptible to virus infection was greater, as had already been observed in previous work,[2] by using most of the upper epithelial layer together with the lower layers.

The results of 13 serial passages in which the entire tissue was used in this manner indicated a trend toward an increased titer, as shown in table 1. Although contaminated cultures occasionally occurred, the antibiotics were quite effective in controlling bacterial growth. The entire epithelial cover of the bovine tongue, therefore, can be used satisfactorily in virus propagation, giving a method for economical harvest of tongue epithelium for vaccine production.

The effect of increasing the content of cysteine hydrochloride in the cultivating medium was evaluated by doubling and tripling the amount utilized in Baker's fluid. Thus, mediums containing 180 and 270 mg. of cysteine hydrochloride per liter were compared with Baker's fluid containing the standard 90 mg. per liter. The results of this experiment, as shown in table 1, demonstrated little or no value in doubling the amount of cysteine, and tripling the amount appeared to be somewhat detrimental to virus propagation. Metabolism studies with a Warburg apparatus[4] indicated that the level of cysteine hydrochloride in the medium was optimal at 120 mg. per liter.

An epithelial extract made by grinding and pressing the keratinous upper layer of the lingual epithelium was sterilized by filtration and added to cultures in a series of four passages. The final passage, on bovine titration, showed no advantage over those cultures not containing the extract. This would indicate that probably no catalytic factor had been extracted, at least not in a form utilizable in the culture.

TABLE 1

Portion of lingual epithelium used	Approx. quant. (Gm.)	Medium	No. passages made	Strain of Vallée type A virus	Titers obtained in cattle
Lower layer only*	4	Baker's fluid	14	European	1 : 218,000 1 : 245,000 1 : 395,000 1 : 395,000 1 : 193,000
Entire epithelium**	24	Baker's fluid	13	European	>1 : 1,000,000 >1 : 1,000,000 1 : 460,000 1 : 400,000 1 : 700,000
Entire epithelium	24	Baker's fluid with 18.0 mg. per 100 mg. cysteine hydrochloride	5	European	1 : 745,000 1 : 315,000
Entire epithelium	24	Baker's fluid with 27.0 mg. per 100 mg. cysteine hydrochloride	5	European	1 : 257,000 1 : 420,000 1 : 242,000
Lower layer only	4	Baker's fluid with epithelial extract	7	European	1 : 315,000
Lower layer only	4	Tyrode's solution with 10% serum, 9 mg. per 100 mg. cysteine, 10% epithelial extract	8	European	1 : 395,000
Entire epithelium	24	Tyrode's solution with 10% serum and 9 mg. per 100 mg. cysteine hydrochloride	8	European	1 : 1,000,000 1 : 315,000 1 : 395,000
Upper layer only***	60	Baker's fluid with 12.5 mg. per 100 mg. cysteine hydrochloride	8	Mexican MP	1 : 500 >1 : 100
Lower layer only	16	Baker's fluid with 12.5 mg. per 100 mg. cysteine hydrochloride	8	Mexican MP	>1 : 10,000 1 : 31,500 <1 : 10,000 1 : 150,000
Entire epithelium	48	Baker's fluid with 12.5 mg. per 100 mg. cysteine hydrochloride	2	Mexican MP	1 : 760,000 1 : 462,000
Entire epithelium	48	Baker's fluid with 12.5 mg. per 100 mg. cysteine hydrochloride, 18 mg. per 100 mk. lysine, 5.0 mg. per 100 mg. arginine, 300.0 mg. per 100 mg. peptone, and no serum	2	Mexican MP	1 : 920,000 1 : 515,000

*Sterile deep layer of epithelium. **Both sterile deep layer and the superficial keratinous epithelial layers. ***Superficial keratinous epithelial layer.

The epithelial extract was added to another series of cultures in which Fischer-Tyrode's solution, containing 9 mg. per 100 mg. of cysteine and 10 per cent normal bovine serum, was used as a basic medium. The tissue used was only the live deep layer of the tongue epithelium. A companion series differed in that no epithelial extract was added and the entire epithelium, including the keratinous superficial layer, was the tissue used.

The results, as presented in the accompanying table, showed that the virus could be cultivated in the absence of a medium as complex as Baker's fluid, but a more optimum condition appeared to be present when Baker's fluid was used in conjunction with the entire epithelial layer of the tongue.

Several attempts to cultivate the Mexican MP strain of Vallée type A foot-and-mouth disease virus had previously been made with limited success. A series of cultures was prepared to determine the strain's possible affinity for a portion of the epithelial tissue and also to determine the possibility of further modifications of the medium.

One series of eight culture passages, in which only the keratinous superficial layer of the lingual epithelium was used, demonstrated only weak propagation of the virus, with titrations of 1 : 500 or less. A second series of eight culture passages, in which only the live lower layer of the epithelium was used, gave better results but was not impressive, the highest titer being somewhat less than 1 : 200,000.

In a third series of two cultures, the entire epithelium, including the keratinous layer, was used with much more success. Initial titers were 1 : 760,000 and 1 : 462,000. A fourth series of two passages, also utilizing the entire epithelium, incorporated medium changes suggested by the metabolism studies. These changes included additions of 180 mg. of lysine, 50 mg. of arginine, the reduction of peptone to 3,000 mg., and the elimination of serum. Initial results again looked promising; titers of 1 : 920,000 and 1 : 515,000 being obtained.

SUMMARY AND CONCLUSIONS

The results showed that the entire epithelium, including the keratinized superficial layer of the bovine tongue, can be used satisfactorily in the propagation of the foot-and-mouth disease virus. The increase of cysteine hydrochloride had no conclusive benefit on the cultivating process. It was found that foot-and-mouth disease virus could be cultivated in a medium other than Baker's fluid, but titers of virus grown in Baker's fluid medium were higher. When extracts of the keratinous superficial layer of the lingual epithelium were added to the medium, little benefit was noted.

Mexican MP (Mexican Puebla) strain of Vallée type A virus was cultivated in cultures in which the entire epithelium was used. Little success accompanied trials in which either upper or lower layers of epithelium alone were used with this virus strain. Cultures in which there were additions of arginine and lysine, elimination of serum, and reduction of peptone in the medium maintained high titer. However, evidence was insufficient to state that the modifications were advantageous.

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Susceptibility of Cattle to Brucella Suis Following Vaccination with Brucella Abortus Strain 19

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THE SUSCEPTIBILITY of cattle to *Brucella suis* has received some attention by research workers. It is definitely known that dairy cattle do become infected with the porcine strain of Brucella and, when such infection occurs in a raw milk supply, multiple cases of human brucellosis result. Borts and co-workers[1] traced an epidemic of undulant fever to *Br. suis* in a raw milk supply. Hayes,[2] Hasseltine,[3] and Beattie and Rice[4] have all reported the isolation of living *Br. suis* from cow's milk. From previous work at the Purdue University Agricultural Experiment Station, it was reported[5] that active infection followed intramammary exposure of dairy cows to *Br. suis*. A search of the literature has failed to disclose any reports relative to the effect of *Brucella abortus* strain 19 vaccination on subsequent exposure to *Br. suis*. This report deals with the results of exposure of dairy cattle to *Br. suis* following vaccination with *Br. abortus* strain 19.

Experimental Methods

Four 6-month-old female calves were vaccinated with *Br. abortus* vaccine (strain 19) according to the recommendations of the manufacturer. Tests were conducted at irregular intervals to determine the postvaccination blood serum-agglutination response. The standard plate method was employed, using standard antigen as prepared by the U. S. Bureau of Animal Industry at serum dilutions 1 : 25, 1 : 50, 1 : 100, 1 : 200, and 1 : 400.

The animals were bred at approximately 15 months of age. After calving, the animals were bred back and observed for a period of three months, during which several milk samples were obtained and examined for the presence of Brucella. Approximately three months after calving, the animals were exposed to brucellosis by injecting approximately 31,000,000 viable *Br. suis* organisms into the right front quarter of each animal.

Blood was collected at weekly intervals, postexposure, to determine serum-agglutination response and examined for the presence of Brucella organisms. Milk from each quarter was examined by direct culture methods at irregular intervals beginning on the fourteenth day after exposure to *Br. suis*. Approximately eight weeks after exposure to *Br. suis*, the animals were destroyed and examined by direct culture methods for the presence of Brucella.

Results

All animals showed a variable blood serum-agglutination response following vaccination with strain 19 vaccine. One of the heifers died suddenly of undetermined cause at approximately 14 months of age. A second heifer failed to conceive after repeated breeding and was sold for slaughter at approximately 27 months of age.

The 2 remaining animals calved normally. Direct culture of fetal membranes and the colostrum failed to disclose the presence of Brucella in either animal. Several subsequent cultural examinations of the milk from each of these animals were negative for Brucella organisms.

Subsequent to exposure to *Br. suis*, both animals showed rather marked swelling of

TABLE I—Serum-Agglutination Response Following Vaccination with Brucella Abortus Strain 19

Animal (No.)	Prevaccinal titer	Agglutination response (weeks after vaccination)			
		7	32	64	96
16	———	+++P—	++PT—	+PT—	+PT—
17	———	++———	———	———	‡
18	———	+++——	PT———	T———	———
19	———	+++T—	*	.....	.....

*Died (cause not determined); ‡ = sold for slaughter; + = complete agglutination; P = partial agglutination; T = trace of agglutination; — = negative.

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the exposed quarters. The right front quarter of cow 16 showed the more acute reaction, with almost complete cessation of milk flow beginning on the third day following exposure and lasting for several days, after which the inflammatory reaction gradually subsided and milk secretion returned to normal. *Brucella suis* was recovered intermittently from the milk of the exposed quarter up until the time of slaughter, two months after exposure. The exposed quarter of cow 18 showed some swelling and *Br. suis* was recovered from the exposed quarter on the forty-ninth day after exposure. Following intramammary exposure to living *Br. suis*, blood serum-agglutination responses indicative of infection were elicited in both animals. *Brucella suis* was not recovered from the blood of either animal during the course of the trial.

At autopsy, *Br. suis* was recovered from the right front quarter of cow 16 and from the right supramammary lymph nodes of both animals by direct cultural methods.

TABLE 2—Serum-Agglutination Response Following Exposure to Brucella Suis

Animal (No.)	Preëxposure titer	Agglutination response (days after exposure)				
		15	23	42	53	63
16	+ P T — —	+ + + T T	+ + + + +	+ + + T T	+ + P P T	+ + P T T
18	— — — — —	+ + + T T	+ + + + +	+ + + + +	+ + + P T	+ + P T —

+ = complete agglutination; P = partial agglutination; T = trace of agglutination; — = negative.

SUMMARY

1) Two heifers vaccinated with *Brucella abortus* strain 19 at 6 months of age were exposed, following calving, to brucellosis by injecting a suspension of *Brucella suis* into the teat canal. Both became infected, as measured by blood serum-agglutination response and recovery of *Br. suis* from the milk during life and from the tissues at autopsy.

2) Thus, such vaccination did not protect these 2 bovine animals from such exposure to brucellosis.

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Effect of Massive Doses of Penicillin and Dihydrostreptomycin, Employed Singly or in Combination, on Staphylococcus Pyogenes Mammary Infections

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THIS PAPER is concerned with a single phase of studies on the control of infections of the mammary gland in a large, self-contained dairy herd. To summarize briefly what has been accomplished as a result of these investigations from 1943 on, the initial study was devoted to *Streptococcus agalactiae*, which was found in the milk of approximately 35 per cent of a herd of about 200 cows. Through the establishment of a program of segregation and therapy, this organism was eradicated in two years. Attention was then turned to infections of the udder caused by *Staphylococcus pyogenes*.

Serious clinical mastitis attributable to *Staph. pyogenes* had not been common in the herd, although, in the early period of investigation (that is, during the third quarter of 1945), 52 per cent of 226 cows had shed staphylococci in the milk. The program of control initiated against this infection and pursued diligently throughout the succeeding five years proved fruitful, since, in the last quarter of 1950, only 17 (6%) of the 276 animals comprising the herd were shedding *Staph. pyogenes*. Control measures consisted of complete segregation of all shedders of staphylococci, application of chlorine to the teats of all cows both before and after each milking (since 1947), and intensive treatment of infected lactating and dry quarters with penicillin. Single infusions of penicillin were dispensed with early in the study as being inadequate, and thereafter the penicillin was administered in multiple doses at 12-, 24-, or 48-hour intervals. Up to the end of 1948, the maximum total dosage of penicillin, administered either to lactating or dry quarters, was 400,000 units, and results of therapy to this time have been published.[1] To restate these results concisely, 28 per cent of 123 lactating quarters and 67 per cent of 184 dry quarters were freed of *Staph. pyogenes* on first treatment with penicillin. Because of the poor response in lactating quarters, penicillin therapy was limited to dry quarters during 1947 and 1948.

The yearly rate of new infections remained rather constant, averaging 14 per cent. On the other hand, the rate at which infected cows became negative for *Staph. pyogenes* declined, so that in the third and fourth years of the control program a greater number of cows became shedders of this pathogen than became nonshedders. Several factors apparently contributed to this unfavorable outcome. The percentage rate of new infections, although remaining constant, was operative among a rapidly increasing number of clean cows, so that the actual number of shedders of *Staph. pyogenes* rose each year. These new infections usually made their initial appearance in an udder during lactation, and since the administration of therapy to infected lactating quarters was discontinued in the last two years, such new infections had an opportunity to become well established before treatment was attempted in the next dry period.

The status of the herd at the beginning of 1949 suggested that further significant reduction in incidence of mammary gland infection with *Staph. pyogenes* could be realized only through: (1) increasing the rate of culling cows refractory to treatment, (2) improving the effectiveness of therapy by increasing dosages and by employing antibiotics in combination, and (3) introducing additional managerial features in an attempt to lessen the occurrence of new infections. Consideration of these possibilities indicated that attempts at improvement of therapy would be the most feasible. The purpose of this paper is, therefore, to present a summary of the results obtained

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from the use of massive doses of penicillin and/or dihydrostreptomycin for the treatment of recent as well as long-standing infections.

METHODS

Complete details on the methods employed in this herd have been published.[1] In brief, milk samples for bacteriological study were drawn from a portion of the herd at weekly intervals. An attempt was made to sample each cow at least once every six weeks. Under this system of sampling, it was possible for an udder to become infected with *Staph. pyogenes* shortly after having provided a negative sample and, in such a case, six weeks could have elapsed before the infection was detected. Composite milk samples were drawn on routine test from cows having a negative history of infection. When *Staph. pyogenes* occurred in the milk for the first time, individual quarter samples were drawn the following week and, if the pathogen was found again, the cow was classified as infected. Beginning in 1949, such new infections usually were treated immediately, irrespective of whether the udder was lactating or dry. In lactating glands, the interval between infusions was usually shorter than when infections in dry quarters were being treated. The penicillin was usually crystalline penicillin G potassium purchased on the open market by bid; the dihydrostreptomycin was furnished for this study by Merck and Company, Inc., Rahway, N. J. These antibiotics were used separately or in combination and were administered by infusion in 50 ml. of sterile distilled water. The term treatment, as used in this paper, refers to a series of infusions administered at 12-, 24-, or 48-hour intervals. After treatment, several consecutive weekly samplings were made and, if the infection persisted, further treatment was usually attempted. Treated quarters that remained free of *Staph. pyogenes* for a period of three months were regarded as having been cured. In dry udders, this period was lengthened to include three months after the day of freshening, since experience had shown that some dry quarters, which appeared to be negative after treatment, would, upon freshening or shortly thereafter, commence to shed again.

RESULTS

Penicillin and dihydrostreptomycin were employed singly or in combination in a variety of dosage patterns. The treatments given to lactating quarters are presented in table 1; those administered to dry quarters are summarized in table 2. Each table shows only the results from first application of a specific pattern of treatment; this includes retreatment with a pattern differing from a previous unsuccessful one. Thus, each specific treatment pattern can be evaluated from the results obtained against *Staph. pyogenes* upon first application.

In table 1, 57 treatments are shown. These were administered during lactation to 45 separate quarters among 32 cows. Twelve infections were treated with penicillin in total doses per quarter varying from 1.5 to 4 million units; four (33%)

TABLE 1—Results from Treatment of Staphylococcus Pyogenes Infections in Lactating Quarters with Massive Doses of Penicillin and/or Dihydrostreptomycin Employed Singly or in Combination

Treatment pattern*					Duration of infection and No. quarters cured or not cured**									
Single infusion dose	No. infusions in series	Interval between infusions (hr.)	Total quantity of antibiotic administered		6 weeks or less		2-6 months		7-12 months		Over 12 months		Total treatments	Cured (%)
			P	D	C	NC	C	NC	C	NC	C	NC		
P500M	3	24	1,500M	0	1	..	..	..	..	..	..	..	..	...
P500M	4	48	2,000M	0	..	1	..	..	..	..	..	..	..	...
P500M	8	12	4,000M	0	..	1	..	1	..	..	..	..	..	...
P1,000M	3	24	3,000M	0	..	..	1	..	..	..	..	..	..	...
P1,000M	4	24	4,000M	0	2	1	..	..	..	..	..	4	12	33.0
D1 Gm.	2	12	0	2 Gm.	..	1	..	..	..	..	..	..	..	...
D1 Gm.	3	24	0	3 Gm.	..	..	..	1	..	..	..	..	..	...
D½ Gm.	10	12	0	5 Gm.	..	..	..	..	1	..	..	..	..	...
D1 Gm.	6	12	0	6 Gm.	..	..	..	..	..	1	..	..	..	...
D1 Gm.	8	12	0	8 Gm.	..	..	..	1	..	..	..	..	5	20.0
P1,000M D1 Gm.	3	48	3,000M	3 Gm.	2	1	..	..	..	..	..	..	..	...
P1,000M D1 Gm.	4	24	4,000M	4 Gm.	8	1	3	..	2	2	1	3	..	...
P1,000M D1 Gm.	5	24	5,000M	5 Gm.	2	1	4	..	3	..	1	1	..	...
P1,000M D1 Gm.	6	24	6,000M	6 Gm.	1	..	..	1	..	..	..	..	..	...
P1,000M D1 Gm.	8	12	8,000M	8 Gm.	1	..	..	..	..	..	..	1	..	...
P2,000M D1 Gm.	5	24	10,000M	5 Gm.	1	..	..	..	..	..	..	..	40	73.0
Totals					18	7	8	4	6	3	2	9	57	...
Percentage cured					72	..	67	..	67	..	18	..	..	60.0

*P = penicillin; M = 1,000; D = dihydrostreptomycin; Gm. = gram. **C = cured; NC = not cured.

were cured. Dihydrostreptomycin alone was employed on five infections in total doses of 2 to 8 Gm., and one quarter (20%) ceased to shed. A combination of these antibiotics was administered to 40 quarters employing doses of penicillin ranging from 3 to 10 million units and dihydrostreptomycin from 3 to 8 Gm. Twenty-nine of the quarters (73%) so treated became negative. Results with all treatments in lactating quarters gave the following cures: 72 per cent, among infections of less than six weeks' duration; 67 per cent, in infections of two to twelve months' duration; and 18 per cent among infections of more than one year's duration.

In table 2, 50 treatments are summarized. These were given to 44 dry quarters among 32 cows. On 26 infections, penicillin alone was used in total doses ranging from 1.5 to 4 million units, and 17 quarters (65%) became negative. Dihydrostreptomycin alone was used in 2 cases only, and each was cured by a total dose of 4 Gm. The two antibiotics were combined in the treatment of 22 infections, using total doses of 2 to 8 million units of penicillin and 4 Gm. of dihydrostreptomycin; following treatment, 16 quarters (73%) no longer shed. Cures were effected in all infections of less than six weeks' duration, in 80 per cent of the infections of two to six months' standing, and in 71 per cent of infections of seven to twelve months' duration. Among infections of longer standing, however, only 38 per cent responded.

Infections which persisted following initial exposure to penicillin and/ or dihydrostreptomycin in massive doses were retreated and, if the infection failed to respond to the second attempt with therapy, further treatments were usually administered until a cure was obtained or the cow was removed from the herd. The results from repeated treatments in refractory infections are summarized in table 3. Fourteen quarters were involved of which seven had been infected from two to eight months when treatments with massive doses were begun; the remaining seven quarters had been infected from thirteen to forty-two months. Most of the quarters in the latter group had been subjected to therapies of various kinds prior to those reported here. Among these 14 quarters given repeated treatments, nine were classified as cured following two to seven treatments, although two quarters shed *Staph. pyogenes* again after having been negative for four and five months, respectively. In the cured cases, the total quantity of penicillin administered ranged from 4 to 22 million units and that of dihydrostreptomycin from 4 to 39 Gm. These treatments were distributed over periods ranging from one to sixteen months, with an average duration of seven months per quarter. Five quarters continued to shed following either three or six separate treatments with massive doses. In these cases, the total quantity of penicillin infused varied from 12 to 23 million units and that of dihydrostreptomycin from 8 to 23 Gm. The period of time covered by the treatments extended from two to twenty

TABLE 2—Results from Treatment of Staphylococcus Pyogenes Infections in Dry Quarters with Massive Doses of Penicillin and/or Dihydrostreptomycin Employed Singly or in Combination

Treatment pattern*					Duration of infection and No. quarters cured or not cured**									
Single infusion dose	No. infusions in series	Interval between infusions (hr.)	Total quantity administered P	Total quantity administered D	6 weeks or less C	6 weeks or less NC	2-6 months C	2-6 months NC	7-12 months C	7-12 months NC	Over 12 months C	Over 12 months NC	Total treatments	Cured (%)
P500M	3	48	1,500M	0	2	..	2	1	3	..	2	2	..	...
P500M	4	24	2,000M	0	..	..	..	..	..	..	..	1	..	...
P500M	4	48	2,000M	0	1	..	1	..	2	..	1	1	..	...
P300M	4	24	3,600M	0	..	..	1	..	1	..	..	..	..	...
P1,000M	3	48	3,000M	0	..	..	..	..	1	..	..	1	..	...
P1,000M	4	48	4,000M	0	..	..	..	..	..	2	..	1	26	65
D1 Gm.	4	48	0	4 Gm.	..	..	..	..	1	..	1	..	2	100
P500M D1 Gm.	4	48	2,000M	4 Gm.	1	..	..	..	..	..	1	..	..	...
P1,000M D1 Gm.	4	24	4,000M	4 Gm.	1	..	..	..	..	..	..	2	..	...
P1,000M D1 Gm.	4	48	4,000M	4 Gm.	10	..	..	..	2	2	1	1	..	...
P2,000M D1 Gm.	4	48	8,000M	4 Gm.	..	..	..	..	..	..	..	1	22	73
Totals					15	0	4	1	10	4	6	10	50	...
Percentage cured					100	..	80	..	71	..	38	..	..	70

*P = penicillin; M = 1,000; D = dihydrostreptomycin; Gm. = gram. **C = cured; NC = not cured.

months, with an average of twelve months for each quarter.

DISCUSSION

Of the patterns of treatment used, none has received sufficient trial to permit selection of the most effective method of therapy. However, the results, viewed in their entirety, show definite trends which parallel the results obtained previously in this herd with penicillin alone.[1] A difference, however, is noted in the effectiveness of therapy administered to lactating quarters. Prior to 1949, when penicillin alone was used in total doses not exceeding 400,000 units per treatment, 28 per cent of 123 lactating quarters were cured of *Staph. pyogenes;* whereas, among 57 treatments administered in this study, 60 per cent brought about cures. This improvement in results appears *a priori* to have been brought about by the infusion of penicillin and dihydrostreptomycin in combination. In table 1, it can be seen that, among a limited number of infections treated with either penicillin or dihydrostreptomycin alone, the percentage of cures was 33 and 20, respectively; when the antibiotics were infused in combination, 73 per cent of the treatments produced cures. The infusion of these antibiotics in combination into infected dry mammary glands did not lead to results that would indicate an improvement over the use of massive doses of penicillin alone in the dry udder.

The rate of cures was, in general, inversely proportional to the length of time the infections had persisted, and, in addition, therapy was found to be more effective in dry than in lactating glands. These observations tend to substantiate an hypothesis which was proposed in an earlier publication[1] to explain results previously obtained with penicillin in this herd; namely, that success or failure in freeing the mammary glands of *Staph. pyogenes* infections was related to the stage of pathogenesis rather than to the sensitivity of the organism to the antibiotic. To elaborate upon this hypothesis, it might be suggested that the pathogenesis of this organism in the mammary gland proceeds in three stages. In the first stage, the staphylococci enter the mammary gland by way of the streak canal and, at first, live and multiply in the milk and on the surface of the epithelium. Over a period of time, some of the organisms become localized within the epithelium of the ducts and perhaps to some extent in the epithelium of the alveoli; this represents the second stage of pathogenesis. If invasion of the tissues has not proceeded beyond this stage prior to drying off of the gland, then, during the process of involution, the staphylococci may be released into the lumen of the gland as a result of the involution and sloughing of epithelial cells. Essentially, this would cause a reversion to conditions identical with the first stage of pathogenesis. In infections of long duration, the organisms finally penetrate beyond the epithelium and into the stroma of the gland, constituting the third stage of pathogenesis. Organisms in the stroma would not be affected by involution of the gland and would not, therefore, be released from the stroma. This hypothesis assumes that each infection would eventu-

TABLE 3—Results from Repeated Treatments with Penicillin and/or Dihydrostreptomycin in Refractory Infections of Staphylococcus Pyogenes in the Mammary Gland

Cow	Quarter	Duration of infection (in months) when exposed to massive therapy		No. treatments and result of last treatment**	Total antibiotics infused†	
		Initial treatment	Final treatment		P (million units)	D (Gm.)
1658	RR	2	3	2 C	6.0	4.0
1734	RR	2	11	7 C	16.0	39.0
1734	LF	2	16	6 NC	20.0	23.0
1967	LF	3	19	3 C	13.0	4.0
1017	LR	7	9	3 NC	12.0	12.0
1052	RR	7	8	2 C*	8.0	4.0
1370	LF	8	10	2 C	8.0	8.0
1384	RR	13	28	6 NC	23.0	9.0
931	RF	18	23	2 C	4.0	4.0
1947	RR	20	22	2 C	14.0	14.0
1725	RR	28	40	3 NC	12.0	8.0
1121	RR	41	56	6 C‡	22.0	12.0
1052	LR	41	61	6 NC	21.4	16.0
1017	RR	42	44	3 C	12.0	12.0

*Positive again after four months. ‡Positive again after five months. **C = cured; NC = not cured. †P = penicillin; D = dihydrostreptomycin.

ally progress from the first through the second to the third stage, so that newly established infections are representative of conditions in which the organisms are free living in the lumen of the gland; whereas, the long-standing infections are characterized by deep penetration of the staphylococci into the tissues. Obviously, if these conditions actually exist, fresh infections would perhaps be readily overcome by infused antibiotics. However, long-standing ones would be more resistant to therapy, since it may be further assumed that tissue penetration or localization would, in time, provide some protection to the organisms against infused antibiotics and it is probable that the degree of protection may be related to the depth of tissue penetration.

Infections that are not destroyed by infusions of antibiotics during lactation but are readily overcome by repeating the treatment during the dry phase would, upon the basis of this hypothesis, represent instances in which the staphylococci had penetrated the epithelium but lost their protective position through changes brought about during involution of the gland. Infections that resist many treatments with infused antibiotics would represent instances of deep tissue penetration of the staphylococci. However, since it has been demonstrated that some persistent infections finally disappeared after repeated treatments, the explanation may be that each treatment brings about destruction of some of the deeper foci of infection until finally, after many exposures to massive doses of the antibiotics used either singly or in combination, all of the organisms are eventually reached and destroyed.

CONCLUSIONS

Penicillin and dihydrostreptomycin were employed singly or in combination in a variety of treatment patterns, but none was given any considerable number of trials. The method employed most frequently consisted of a combination of 1 million units of penicillin and 1 Gm. of dihydrostreptomycin in 50 ml. of sterile distilled water infused four times at 24-hour intervals in lactating glands, and at 24- or 48-hour intervals in dry glands. This combination of antibiotics and method of administration produced cures in 70 per cent of 20 lactating quarters infected with *Staphylococcus pyogenes* and in 73 per cent of 19 dry quarters.

On the whole, the results obtained suggest that massive doses of penicillin and dihydrostreptomycin used in combination are more effective against *Staph. pyogenes* than penicillin alone, especially in the treatment of lactating glands. Furthermore, this combination and dosage of antibiotics should be effective in the treatment of clinical mastitis caused by staphylococci, streptococci, or coliform organisms involved either as single or concurrent infections.

In cases of acute mastitis in which the etiology is not apparent to the practitioner, the use of these combined antibiotics, in the previously mentioned massive doses, is encouraged whenever it is economically feasible. This quantity of penicillin should be more than sufficient to eliminate streptococci; the dose of dihydrostreptomycin (or streptomycin) should be adequate for the less severe cases of mastitis due to coliform organisms;[2] and the combination of antibiotics should be more effective against *Staph. pyogenes*, as shown in this study, than either of the antibiotics used alone.

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Fractionation of the Calcium in Bovine Blood by Fluoride Precipitation

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IT IS WELL known that soluble fluorides precipitate calcium from blood and that small amounts of sodium fluoride, sufficient to render blood incoagulable, may not remove all the calcium from the plasma. Further study of this question revealed that certain anions (e.g., bicarbonate, citrate) appear to protect calcium from precipitation by sodium fluoride, as compared to others that do not (e.g., chloride, phosphate, oxalate). Applying this principle to bovine blood, it was found that definite quantities of sodium fluoride precipitate comparatively constant portions of calcium from the blood of normal animals. Moreover, this fluoride-precipitable portion varies widely under conditions known to affect total blood calcium level, such as parturition, changes in feeding, milk fever, and ketosis.

LITERATURE

The calcium in blood is generally regarded to be present in several forms, some physiologically active and some inert. Total blood calcium, the figure generally reported, does not take into account differences in proportions between active and inert forms, although obviously such differences may be essential to interpreting the calcium level. Since 1913, when Rona and Takahashi introduced fractionating serum calcium by dialysis,[1] this concept has gained favor with physiologists and clinicians alike. Refinements in the process yielding more critical results have been suggested by Greenberg and Gunther (ultrafiltration)[2] and by Greene and Powers (vivadialysis).[3] Using a different approach, McLean and Hastings[4] introduced the direct measurement of the calcium ion in serum, employing the reactivity to calcium of the isolated frog heart.

Some studies were reported on bovine blood fifteen to twenty years ago, but interest in this aspect of the calcium problem has not been sustained. Part of the reason for this apparent indifference may lie in the complexity of the methods employed, although this must be coupled with failure to demonstrate important advantages from their use. Unfortunately, the comparatively simple direct calcium ion tritration seems doomed for use on cattle blood, because the frog heart does not respond satisfactorily to bovine serum.[4] Actually, apart from gross changes in total blood calcium, such as occur at parturition and particularly in milk fever, there has been little progress toward correlating changes in bovine blood calcium with physiological disturbances, where such changes are suspected.

Sjollema and Seekles, by ultrafiltration, recovered 4.50 to 6.39 mg. of diffusible calcium per 100 ml. of blood (mean, 5.23) from normal cows, representing 48 to 66 per cent of the total calcium (mean, 56). Cows with milk fever had 0.68 to 4.50 mg. of diffusible calcium per 100 ml. of blood (mean, 2.36), equal to 25 to 100 per cent of the total calcium (mean, 56). Applying a technique of oxalate precipitation upon these filtrates, they obtained 1.47 to 2.00 mg. of the calcium ion per 100 ml. of blood (mean, 1.65) from normal cows, and 0.35 to 0.70 mg. of the calcium ion per 100 ml. of blood (mean, 0.44) in milk fever.[5] In a later report from the same laboratory, they showed that, with the approach of parturition and onset of lactation, the downward trend in total blood calcium is reflected in a similar trend in diffusible calcium, but with a larger proportion of the lower total calcium levels recovered in the diffusible fraction. In 1 cow which failed to develop milk fever, the diffusible calcium rose from 4.5 mg. at parturition to 8.0 mg. per 100 ml. of blood one hour later, although total calcium remained unchanged at 8.6 mg. per 100 ml. of blood. This effect was transient, however, for five hours later, diffusible calcium had relapsed to 3.7 mg. per 100 ml. of blood out of a total calcium of 8.5 mg. per 100 ml. of blood.[6] These observations are particularly interesting in comparison with our results employing sodium fluoride precipitation (*see* table 5).

Generally comparable results were obtained by Little and Mattick,[7] who reported studies including diffusible blood calcium data from a series of normal parturitions and cases of milk fever. In contrast to Seekles *et al.*,[5] however, their normal cows at parturition showed a somewhat greater fall in diffusible calcium than in total calcium. Otherwise, their results were generally comparable. Godden and Duckworth[8] used a $BaSO_4$-adsorption technique to fractionate blood calcium. They found the cal-

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cium in blood from normal nonparturient cows to be about evenly divided between adsorbable and nonadsorbable fractions. When reductions occurred in total calcium, as in normal parturition and milk fever, they affected the adsorbable calcium predominantly, although some reduction followed in the nonadsorbable fraction.

METHODS

For the precipitation of calcium from simple solutions, dried sodium fluoride, c.p. grade, was weighed carefully on an analytical balance and transferred to clean, dry, 15-ml. centrifuge tubes. Duplicate tubes were prepared, containing 2.5, 5.0, 10.0, 15.0, and 20.0 mg. of sodium fluoride, equal to 0.5, 1.0, 2.0, 3.0, and 4.0 mg. per milliliter when 5 ml. of solution were added. More consistent results were obtained when dry sodium fluoride was used rather than a solution; in the case of blood, sodium fluoride solutions were completely unreliable.

Simple calcium solutions were prepared as indicated in table 1 and adjusted to pH 7.3, except $CaCl_2$ (A). Then, 5.0 ml. of solution were drawn up into a pipette and blown rapidly into the bottom of the fluoride-containing tube, which was immediately stoppered and shaken vigorously for several seconds. This procedure was repeated through the entire series of ten tubes, using the same solution. Duplicate series were prepared for the other solutions, some incomplete. After standing at least fifteen minutes, the tubes were again shaken momentarily, stoppers removed, and they were centrifugalized fifteen minutes at high speed (2,000 to 3,000 r.p.m.). Whereupon, 2 ml. of the supernatant were transferred from each tube to a new tube and examined for calcium following the Tisdall method.[9] Simultaneously, total calcium was determined on the untreated solution.

A similar process was employed on blood. Serum and heparinized plasma were tried at first, following precisely the method described for simple solutions. Later, heparinized whole blood was used. It was chosen for routine analyses, because it gave more consistent results than serum or plasma. In the case of whole blood, however, a ratio of 7:4 was applied to correct for cell volume. On the basis of a red cell volume averaging 40 per cent, 7 ml. of whole blood were assumed to approximate 4 ml. of plasma.

Routinely, about 50 ml. of blood were collected in a tube containing heparin, with a minimum of obstruction to flow in the vein. After shaking or inverting the specimen several times, 7 ml. were withdrawn into a pipette and blown into each of duplicate clean, dry centrifuge tubes containing 12 and 16 mg. of sodium fluoride, equal to 3 and 4 mg. per milliliter of plasma. After promptly stoppering and shaking a full minute, the four tubes were set aside for fifteen minutes, then shaken again momentarily, the stoppers removed, and the tubes centrifugalized fifteen minutes at high speed. The remainder of the untreated blood was also centrifugalized, after which 2 ml. of plasma were withdrawn from each tube and analyzed for calcium.

Inorganic phosphorus was determined on the plasma by the method of Fiske and Subbarrow.[10] Plasma proteins were computed from specific gravity, determined by the falling drop method,[11] and fibrinogen from the difference between plasma and serum protein levels. Ketone bodies were determined by the method of Behre,[12] and blood sugar according to Folin and Wu.[13]

RESULTS

The addition of sodium fluoride, 0.5 to 2.0 mg. per milliliter, caused complete precipitation of calcium from simple aqueous solutions of chloride, phosphate, ox-

TABLE 1—Sodium Fluoride Precipitation of Calcium from Various Salts in Simple Aqueous Solution

Specimen	Anion (millimols per liter)*	Total** calcium (mg. per 100 ml.)	Calcium remaining in solution (mg. per 100 ml.)** NaF (mg. per ml.) 0.5	1	2	3	4
A	Chloride, 2.3	9.1	0.0	0.0	0.0	0	0
B	Bicarbonate, 2.7	10.9	1.4	1.4	0.0	0	0
C	Phosphate, 1.3	11.2	0.0	0.0	0.0	0	0
D	Citrate, 0.3	10.9	5.2	5.4	5.5	0	0
E	Citrate, 1.0	10.9	9.7	9.7	9.3	0	0
F	Citrate, 3.0	10.9	10.8	10.1	10.0	0	0
G	Oxalate, 0.5	11.4	..	0.0	0.0	0	0
H	Oxalate, 4.0	7.2	..	0.0	0.0	0	0
I	β-hydroxy butyrate, 4.0	6.0	..	0.0	0.0	..	..
J	Gluconate, 2.9	11.7	..	1.4	0.0	..	..
K	Borogluconate, 2.9	11.7	..	10.3	9.9	..	..

*All solutions except A were adjusted to pH 7.3 with M/10 $NaHCO_3$; solutions A to I were prepared from M/10 $CaCl_2$, to which was added either NaH_2PO_4 (C), Na_3 citrate (D to F), Na_2 oxalate (G, H), or β-hydroxy butyrate (I); J and K were prepared from calcium gluconate, U.S.P., in the case of K with the addition of H_3BO_3, 2.1 millimols per liter.

**Total calcium levels were determined analytically (Tisdall method), as was the unprecipitated calcium which remained in solution following the addition of sodium fluoride in the concentrations indicated.

TABLE 2—Sodium Fluoride Precipitation of Calcium from Bovine Blood

Conditions	Calving (days)*	Total calcium (mg. per 100 ml.)	Calcium remaining in plasma (mg. per 100 ml.)** NaF (mg. per ml.) 1	2	3	4	5	6	7	Calcium partition ("free"-"bound")†
1—Normal	—21	10.9	8.9	5.9	4.9	5.1	5.1	4.1	..	5.9-5.0
2—Normal	+ 1	8.5	5.7	4.4	3.3	2.4	1.8	1.2	..	5.7-2.8
3—Normal	0	7.2	6.0	4.0	3.3	3.2	2.9	2.5	0.3	4.0-3.2
4—Normal A	0	6.6	6.2	0.0	0.0	0.0	..	..	..	6.6-0.0
Normal B	+ 1	7.1	6.4	4.0	2.6	2.3	..	2.2	..	4.7-2.4
Normal C	+ 2	7.3	6.3	4.3	2.9	2.5	1.5	1.9	..	4.6-2.7
5—Ketosis	+14	9.1	8.0	5.9	4.7	4.4	3.4	2.3	0.3	4.6-4.5
6—Normal Steer	....	9.4	8.2	5.2	3.9	..	3.9	2.1	1.0	5.5-3.9

*Days before parturition (—), days after parturition (+), and day of parturition (0).
**Results of analyzing plasma (Tisdall method) after the addition of sodium fluoride to heparinized blood and centrifugalizing; for determining amounts of sodium fluoride, assume that 7 ml. of whole blood equals 4 ml. of plasma.
†"Bound" calcium is the mean of calcium remaining in plasma after addition of 3 and 4 mg. of sodium fluoride per milliliter; "free" calcium is total calcium—bound calcium.

TABLE 3—Calcium in Bovine Blood, as Fractionated by Fluoride Precipitation

Conditions*	Feeding**	Specimens (animals)	Plasma calcium (mg. per 100 ml.)†	Total	"Free"	"Bound"
A—Normal, nonparturient	Stable	26/11	mean	10.0	5.8	4.2
			range	9.0–10.9	5.2–6.3	3.0–5.0
B—Ketosis	Stable	5/ 5	mean	9.1	4.5	4.6
			range	7.8–10.2	4.1–4.9	3.7–5.4
C—Milk fever	Stable	11/11	mean	4.5	3.0	1.5
			range	2.9– 5.6	1.6–4.0	0.9–2.1
D—Normal, nonparturient	Pasture	13/11	mean	10.6	5.2	5.4
			range	9.6–11.3	4.3–5.8	4.4–6.0

*Groups A and D include lactating and nonlactating cows and 1 Guernsey steer.
**Groups A, B, and C were examined between Jan. 1 and April 15, 1951, while the cattle were being fed in the stable, on concentrates, hay, and ensilage, but no pasture; group D specimens were collected between April 15 and May 1, 1951, when the cattle were grazing on new spring pasture.
†"Bound" calcium was determined by analysis of duplicated plasma specimens after adding 12 and 16 mg. of sodium fluoride, respectively, to 7 ml. of heparinized blood, shaking, and centrifugalizing. Total plasma calcium—bound calcium = "free" calcium.

TABLE 4—Effect of Intravenous Injection of Sodium Citrate, Calcium Gluconate, and Calcium Chloride upon Blood Calcium of Normal, Nonlactating, and Barren Cows

Experiment	Minutes	Effect on cows	Milliliters injected	Plasma calcium (mg. per 100 ml.) Total	"Free"	"Bound"	Phosphorus (mg. per 100 ml. of blood)
1	0	Began injecting sodium citrate	0	9.5	5.6	3.9	5.9
	12	Cow chewing, ruminating actively	200	..	..	..	..
	27	Cow sleepy, staggering	400	9.8	3.5	6.3	5.5
	50	Cow down, dyspneic	950	9.8	2.6	7.2	5.4
2	0	Began injecting sodium citrate	0	9.3	5.5	3.8	4.6
	30	Cow eating avidly	230	..	..	..	..
	37	Cow sleepy	300	..	..	..	..
	46	Cow staggering slightly	350	..	..	..	..
	55	Marked staggering	600	9.4	1.9	7.5	4.1
	67	Cow down, dyspneic	900	9.1	0.5	8.6	3.9
3	0	Began injecting calcium borogluconate	0	9.7	5.1	4.6	..
	10	Completed injection; cow showed slight muscle tremors, salivation, dyspnea	500	..	..	..	..
	13	Cow same	...	32.0	5.3	26.7	..
	130	Cow appeared normal	...	18.7	4.3	14.4	..
4	0	Began injecting calcium chloride	0	10.7	5.5	5.2	..
	10	Completed injection; cow showed marked muscle tremors, salivation, dyspnea	500	..	..	..	..
	13	Cow same	...	26.6	17.1	9.5	..
	130	Cow appeared slightly depressed	...	14.8	5.9	8.9	..

Injection solutions were prepared as follows: (1) Sodium citrate, U.S.P., 7.25 Gm. per 100 ml.; (2) Calcium gluconate, U.S.P., 116 Gm., and boric acid, c.p., 12 Gm. per 500 ml.; (3) Calcium chloride, c.p., anhydrous, 29.2 Gm. per 500 ml.

alate, and β- hydroxy butyrate. Adjusting the reaction to pH 7.3 with bicarbonate resulted in a slight retention of calcium in the solution. Citrate and borogluconate were the only salts to demonstrate strong obstruction to calcium precipitation by fluoride (*see* table 1).

Citrate appears physiologically significant in this role. Following the method of Krog,[14] we encountered serum citrate levels ranging from 0.1 to 0.5 mM. per liter in normal cows. These findings were in agreement with the reports of others using various procedures.[15, 16] Evidently, citrate levels in cattle blood were approximately comparable to solution D in table 1. Calcium borogluconate is of interest in this connection, not as a normal component of blood, but because it enjoys popularity for the intravenous administration of calcium to cows with hypocalcemia.

Comparing the precipitating action of sodium fluoride in blood with that in simple aqueous solution, the most striking difference was in the levels of effective fluoride concentration. The portion of calcium precipitated from the simple citrate solutions was nearly constant at sodium fluoride levels from 0.5 to 2.0 mg. per milliliter, whereas, at 3.0 mg., precipitation was complete (table 1, D to F). In the case of normal bovine blood, however, reasonably constant precipitation was encountered with sodium fluoride concentrations ranging sometimes from 2 to 5 mg. per milliliter of plasma, although generally the most constant results were encountered between 3 and 4 mg. per milliliter. Accordingly, for purposes of comparison, the mean calcium level remaining in the plasma after the addition of 3 and 4 mg. per milliliter was referred to as the fluoride-soluble or "bound" fraction, the remainder being the fluoride-precipitable or "free" calcium fraction. Equine blood collected during the same period failed to show the same type of curve in three studies on different horses. Other animal species were not examined.

Table 3 summarizes findings in normal cattle and in cattle with milk fever and ketosis. All the examinations collated in groups A, B, and C were during the winter and early spring while the animals were fed in the stable. Their ration consisted in mixed dairy concentrates, good legume hay, and corn or grass ensilage, but no pasturage. As indicated in group D, the addition of luxuriant spring pasturage to the ration was accompanied by a distinct rise in the total plasma calcium, largely ascribable to increase in the bound fraction.

The experiments in table 4 illustrate the relationship between variations in these blood calcium fractions and the physical symptoms attributed to alterations in blood calcium. As free calcium fell (experiments 1 and 2), presumably due to conversion to bound calcium by the injected citrate, symptoms of hypocalcemia became evident, leading to depression, incoördination, and eventual paralysis. These extreme effects are characteristic of milk fever, whereas moderate depression is encountered in ketosis. It is interesting to observe a moderate but distinct reduction in inorganic phosphorus, accompanying the fall in free calcium, since hypophosphatemia characterizes clinical milk fever.

Even though citrate caused a fall in free

TABLE 5—Effect of Uncomplicated Parturition on the Blood Calcium Partition Measured by Sodium Fluoride Precipitation

Days*	Cows:	"Free" calcium — "bound" calcium in plasma** (mg. per 100 ml.)				
		285	286	287	290	291
—14		.....	5.9–5.0	5.4–4.2	.....	.....
— 4		.....	6.2–4.0	.....	.....	5.6–5.2
— 3		.....	6.0–4.2	.....	.....	5.6–4.6
— 2		.....	5.4–4.5	.....	.....	5.9–3.9
— 1		.....	5.3–4.6	.....	.....	5.2–4.6
0		6.6–0.0	5.2–4.3	5.0–3.7	6.1–4.3	5.0–3.8
+ 1		4.6–2.5	6.0–2.0	5.6–3.1	6.1–3.7	5.5–3.6
+ 1½		.....	6.2–3.1	5.3–3.4	.....	.....
+ 2		4.6–2.7	6.2–3.4	5.2–3.6	5.4–3.8	8.5–0
+ 2½		.....	.....	.....	.....	5.1–3.3
+ 3		5.9–2.4	.....	.....	.....	5.1–4.4
+ 7		.....	.....	5.2–4.4	.....	.....
+15		4.9–4.7†	.....	.....	5.4–4.3	.....

*Days before parturition (—); after parturition (+); day of parturition (0). **See text for method: total plasma calcium (not given above) = "free" calcium + "bound" calcium. †Appetite poor (ketosis?).

calcium, the injection of $CaCl_2$ failed to reduce bound calcium. From experiments 3 and 4, it is clear that both borogluconate and chloride caused a distinct rise in the bound fraction. This rise following calcium borogluconate was anticipated in view of the effects observed in simple solution (table 1). The much less marked rise in bound calcium after $CaCl_2$ may be attributed to the acidifying action of this salt, in the light of another experiment in which ethylene diamine dihydrochloride given orally caused a distinct rise in the bound calcium in the same animal. Nevertheless, there was a much greater immediate rise in free calcium accompanying $CaCl_2$ than with calcium borogluconate, where this increase appeared negligible. This difference may account for the observation that the chloride causes more severe physical distress than the borogluconate. In this experiment, muscle tremors and dyspnea were observed in both animals during and immediately following the injections, although these symptoms were less marked with borogluconate than with chloride.

Uncomplicated parturition was attended by significant reductions in both calcium fractions, contributing to the characteristic parturient fall in total blood calcium in the cow. Free calcium fell to a minimum at parturition in 3 of the cows, but bound calcium did not reach its low point until a day or two later (table 5). In 2 cows, bound calcium disappeared completely for a brief time, but it was restored rapidly, and there were no symptoms of physical disturbance. In only 1 of these cows (No. 285) did free calcium fall below 5.0 mg. per 100 ml. of blood. The single study involving a primipara (cow 290), in which neither calcium fraction was significantly reduced, is of interest in view of the infrequence of milk fever in first-calf heifers.

The inorganic phosphorus level in the plasma, which falls characteristically at parturition, appeared in general to vary directly with the bound calcium and inversely with the free fraction, although this relationship was not constant. Moreover, serum protein levels and fibrinogen, both variable but tending to be reduced at parturition, showed no constant relationship with the calcium fractions.

The 5 cows with ketosis (table 3) showed nervous depression manifested by general sluggishness and poor appetite in conjunction with hypoglycemia and ketonemia. At this time, plasma free calcium was below 5.0 mg. per 100 ml. of blood, averaging 4.6 mg. Following clinical recovery, associated with a rise in blood sugar and fall in ketones, the level of free calcium rose to an average of 5.8 mg. per 100 ml. of blood, regardless of whether recovery was attributed to parenteral dextrose therapy, supplementing the ration with effective glycogen formers (e.g., glycerin, tripropionin),[17] or increased intake of feed. In most cases, the rise in free calcium was at the expense of the bound fraction, so that the total plasma calcium varied irregularly.

Discussion

The terms free and bound are used in this connection to distinguish between the portion of plasma calcium free to be pre-

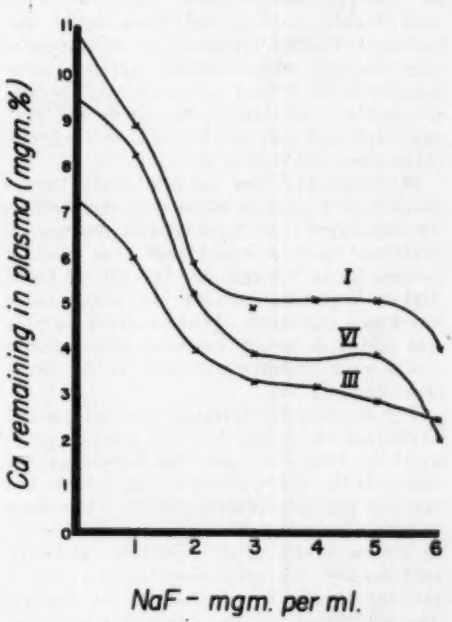

Fig. 1—Calcium remaining in bovine plasma following partial precipitation by varying concentrations of sodium fluoride. Data are taken from table 2, in which the curves are identified by the number of the experiment. The flatness of the curve between 3 and 4 mg. of sodium fluoride per milliliter suggests the use of this concentration as a standard to measure fluoride-precipitable (i.e., "free") calcium. In this sense, fluoride-precipitable calcium is the difference between total calcium (level before adding any sodium fluoride) and the level remaining in the plasma after sodium fluoride precipitation.

cipitated by sodium fluoride and that which is not free, presumably because it is bound inseparably in some soluble form. From the experiments using simple solutions (table 1), it appears unlikely that any of the commonly recognized anions in blood could account for this fixation except citrate. Whether or not other anions are involved, it seems proper to regard the bound calcium fraction as a citrate-like form. Presumably this form is physiologically inert.

To what extent these results obtained by fluoride precipitation can be reconciled to findings by other methods remains to be determined. Seekles, Sjollema, and Van der Kaay[6] recorded disproportionate reductions in diffusible and total calcium at parturition, which suggest similarity between their diffusible fraction and free calcium by fluoride precipitation. In cows with milk fever, Godden and Duckworth[8] encountered marked reduction in their adsorbable calcium, which thereby appears comparable to the bound calcium of this series. Obviously, correlations like these are presumptive and can not be accepted without categorical confirmation.

In comparing free calcium levels by sodium fluoride precipitation with the calcium ion calculated from total calcium and serum proteins,[4] it was found that free calcium ranged up to 1.0 mg. per 100 ml. of blood higher than the calcium ion when whole blood was employed. When sodium fluoride was added to serum, however, free calcium levels were virtually identical to the calculated calcium ion.

The fluoride-precipitation method has the advantage of simplicity. No special equipment is required, and the procedure involves little more effort or skill than the routine calcium determination. For best results, specimens should be examined within a few hours after collection, although refrigerated material may be reasonably satisfactory for twenty-four hours or more. The advantage of this method lies in securing more critical values for blood calcium than are obtainable as the total calcium level, where the inert fraction may vary so widely as to overshadow significant changes in the active fraction.

SUMMARY

1) The precipitation of calcium from simple aqueous solution by soluble fluoride is obstructed by the presence of certain anions (e.g., bicarbonate, citrate, borogluconate). This principle has been applied to the study of calcium in the bovine blood, whereby results are expressed as a fluoride-precipitable or "free" calcium and a fluoride-soluble or "bound" calcium fraction.

2) Intravenous injection of Na_3 citrate into normal cows confirms the implication that this anion may account for variations in the bound calcium, and indirectly also in the free calcium fraction, although obviously it does not exclude the possibility of other "citrate-like" factors.

3) Intravenous $CaCl_2$ causes a profound rise in free calcium, as compared to calcium borogluconate, which appears to raise this fraction very little. This difference seems significant in view of the general impression that, in normal cows, the chloride causes more severe symptoms (*i.e.*, of hypercalcemia) than does the borogluconate.

4) The free calcium fraction, which in normal, stable-fed cattle ranged from 5.2 to 6.4 mg. per 100 ml. of plasma, was reduced to less than 5.0 mg. per 100 ml. in ketosis, and less than 4.0 mg. per 100 ml. in milk fever. These levels reflect the degree of central depression; for cows with ketosis are sluggish and sleepy, whereas milk fever is characterized by coma and prostration.

5) Normal cattle on luxuriant spring pasture showed somewhat higher total plasma calcium levels than cattle fed in the stable on standard rations but without pasturage. This increase is characterized by a distinctly higher bound calcium fraction, reflecting slightly lower free calcium levels than in stable-fed cattle.

6) Limited observations concerning the effects of calving, uncomplicated by milk fever, point to a progressive decline in the free calcium fraction preceding parturition. About the time of parturition, free calcium reverses this trend and rises, sometimes sharply, but at this time bound calcium stages a decline and may disappear entirely for a brief period. Accordingly, it appears that both fractions contribute to the well-known parturient fall in total blood calcium.

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A Critical Study of the Literature on Fluoride Toxicology with Respect to Cattle Damage

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THE OCCURRENCE of fluorosis in cattle is comparatively rare. Damaging fluorosis has been encountered in some cases where cattle were fed mineral supplements containing phosphate rock, which may contain as much as 3½ per cent fluorine. Fluorosis is occasionally (but infrequently) found in cattle where the fluorine content of the drinking water is abnormally high, or in dry, dusty climates, such as in Morocco, where soils containing large amounts of fluorine are heavily deposited on vegetation and consequently ingested by the cattle grazing thereon.

In comparatively recent times, instances have developed (both in this country and in Europe) where emissions into the air of fluorides from industrial operations have been carried by the winds to surrounding land, contaminating the forage and thus causing fluorosis in cattle grazing on these lands.

Fluorosis is commonly defined as "chronic poisoning with fluorine" (American Illustrated Medical Dictionary). A great deal of confusion can result from the use of such a term as fluorosis. To some, the word may mean the condition resulting from *any* ingestion of fluorides above normal levels of intake, and to others, it may mean the condition that results when the abnormal ingestion of fluorides is sufficient to harm the animal (*i.e.*, as the term is defined medically).

It is clear that cattle have a tolerance for fluorine* above the amount normally and naturally absorbed. Consequently, any above normal absorption does not necessarily mean that the consuming animal has been injured. It is therefore the belief of the authors that clearer thinking results if differentiation is made between damaging and nondamaging levels of absorption. In cattle, as in human beings, a slight mottling of the teeth may develop at relatively low levels of fluoride absorption, but the difficulty comes in determining at what point, with a higher than normal absorption, damage to the animal is caused.

In the course of a broad study of the effect of fluorides from industrial operations on cattle, the authors realized the need for some criterion as to whether or not the amounts of fluoride being encountered could be damaging to cattle. As a result, a critical study of the literature was undertaken, reported here, the objectives of which have been threefold:

1) To establish the best field method for the diagnosis of fluorosis in cattle.

2) To determine the borderline between amounts of ingested and absorbed fluorine that will be damaging and nondamaging over an extended period.

3) To establish the best biochemical checks for support of the diagnosis.

THE SYMPTOMS OF CHRONIC FLUOROSIS

The clinical, morbid anatomical, and biochemical findings of fluorosis are fully described by Roholm[33] and others. In diagnosing suspected cases of fluorosis, it is necessary not only to consider all abnormalities observed in the animals in relationship to each other but also to obtain laboratory support for the diagnosis. This is dictated by the following facts:

1) Many of the findings reported in the literature are symptomatic of other maladies as well as fluorosis.

2) A number of symptoms reported in the literature are secondary in nature, dependent on primary effects that must also be present for the secondary symptoms to have any validity, so far as fluorosis is concerned.

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*The element fluorine, because of its extreme chemical activity, never occurs in the free state in nature, but rather always in combination with other elements. Compounds containing fluorine are commonly called fluorides. Therefore, in this paper, the word fluorine refers to the fluorine content of the particular fluoride in question.

TABLE I—Resumé of Experimental Work Done in References Cited in This Manuscript

Investigator	Reference	Animals	Age	Length of experiment	Fluorine compound used	Fluorine in total diet (p.p.m., dry weight basis)	Approx. daily intake of fluorine (mg./kg. of body wt./day)	Resulting conc. of fluorine in dry, fat-free bone (p.p.m.—approx.)	Bone analyzed	Tooth effects	Lameness	Exostoses	Loss in rate of weight gain	Milk loss	Remarks
Agate *et al.*	1	cattle	....	b	b	.....	.....	4,800-10,100	Mandible	Yes	Yes	Yes	Yes	Yes	.....
Agate *et al.*	1	sheep	....	b	b	44–61	.....	4,600-7,700	Mandible	Yes	Yes	Yes	Yes	Yes	.....
A. Am. Feed Off.	2	cattle	....	a	any	68	.....	.....	.....	No (c)	No (c)	No (c)	No (c)	No (c)	.....
Bardelli & Menzani	3	cattle	....	b	b	463–723	.....	1,000-1,320	.....	Yes	Yes	No	No	No	.....
Bardelli & Menzani	3	cattle	....	b	b	463–723	.....	1,260-4,750	.....	Yes	Yes	Yes	Yes	Yes	.....
Blakemore	6	cattle	....	b	b	.....	.....	6,700-16,000	.....	.....	.....	Yes	.....	.....	.....
Blakemore *et al.*	7	cattle	....	b	b	23–91	.....	6,000-16,000	.....	Yes	Yes	Yes	.....	.....	.....
Blakemore *et al.*	7	cattle	....	b	b	11–16	.....	.....	.....	Mottle	No	No	No	No	.....
Elmslie	12	cattle	y	5½ yr.	r.ph.*	43–57	1.59–2.09	.....	.....	sl.	No	No	No	No	.....
Fargo *et al.*	16	swine	....	.....	r.ph.	140	.....	.....	.....	Yes	No	No	No	No	.....
Gaud *et al.*	17	sheep	....	b	b	.....	6.00	1,200-4,400**	Jawbone	Yes	.....	.....	.....	.....	.....
Hatfield *et al.*	20	sheep	y	170 days	r.ph.	.....	3.00	2,000-2,800	Humerus	No	.....	.....	No	.....	.....
Hatfield *et al.*	20	sheep	y	170 days	r.ph.	.....	6.00	4,910	Humerus	sl.	.....	.....	Yes	.....	.....
Kick *et al.*	22	swine	y	160 days	NaF	100	.....	3,200	Femur	No	.....	.....	No	.....	.....
Kick *et al.*	22	swine	....	2 yr.	NaF	200	.....	9,700	Femur	Yes	.....	.....	Yes	Yes	.....
Kick *et al.*	22	swine	....	140 days	r.ph.	100	.....	1,300	Femur	No	.....	.....	No	.....	.....
Majumdar & Ray	30	cattle	s	9 mo.	NaF	.....	0.30–0.40	1,400	Femur	No	No	No	No	No	.....
Majumdar & Ray	30	cattle	s	9 mo.	NaF	.....	3.00–4.00	5,640	Femur	Yes	Yes	Yes	Yes	Yes	Death in 9 mo. (low P diet)
Majumdar & Ray	31	cattle	s	9 mo.	NaF	.....	3.00–4.00	.....	.....	Yes	No	No	No	No	Balanced diet
Meyn & Viehl	38	cattle	....	b	b	75–234	3.40	3,400-5,530	Misc.	Yes	Yes	Yes	Yes	Yes	.....
Mitchell	40	cattle	....	d	d	100	.....	.....	.....	No	No	No	No	No	.....
Mitchell	40	sheep	....	d	d	100	.....	.....	.....	No	No	No	No	No	.....
Mitchell	40	swine	....	d	d	100	.....	.....	.....	No	No	No	No	No	.....
			(Mitchell concludes 100 p.p.m. of fluorine is borderline level in total dry feed above which fluorine damage may occur. For added safety, he recommends 30 p.p.m. of fluorine as tolerance for practical feeding.)												
Ockerse	46	cattle	....	b	b	.....	1.75	.....	.....	Yes	.....	.....	.....	.....	11.78 p.p.m. F in well water
Peirce	48	sheep	y	3 yr.	r.ph.	.....	1.20	5,900	Metacarpal	Yes	No	No	No	No	.....
Peirce	48	sheep	y	3 yr.	r.ph.	.....	2.30	7,000	Metacarpal	Yes	No	No	No	No	.....
Phillips *et al.*	51	cattle	y	4 yr.	r.ph.	57–80	2.30	5,350	Metacarpal	Yes	.....	Yes	No	No	.....
Reed & Huffman	52	cattle	....	5 yr.	r.ph.	158	5.50	.....	.....	Yes	Yes	Yes	Yes	Yes	.....
Slagsvold	58	sheep	....	1 yr.	cryolite	230–250	.....	3,900	Underjaw	No	Yes	Yes	Yes	.....	Anemia
Tolle & Maynard	66	swine	y	4 mo.	ph.l†	276	.....	.....	.....	No	No	No	.....	.....	Deciduous teeth

a—tentative regulations; b—field investigation; c—assumed that Association of American Feed Officials regulations designed to prevent any injury; d—critical evaluation of literature; y—young; s—adult. *rock phosphate; **single analysis of 100 p.p.m. omitted; †phosphatic limestone.

For these reasons, all observed abnormalities in the animals under examination should be evaluated in connection with the unique lesions of fluorosis (which are shown in the following pages to be tooth and bone involvement) and should be checked by laboratory confirmation of the existence of diagnostically significant amounts of fluoride in the forage, the bones, and the urine.

Clinical Findings.—The most sensitive clinical index to fluoride absorption is the mottling, staining, and excessive wearing of permanent teeth that were forming at the time of fluoride ingestion. Practically all investigators, whether concerned with field investigations or controlled feeding experiments, have observed that an effect on the teeth is one of the first indications of fluoride absorption and the manifestation that occurs at a lower level of absorption than other effects.

Elmslie,[12] Evans and Phillips,[13] Fargo *et al.*,[16] Hodges *et al.*,[21] Peirce,[48] Phillips *et al.*,[51] Smith *et al.*,[62, 63] Tolle and Maynard,[66] and others have reported effects of fluoride on teeth when fed at low levels, with no other effects appearing. Teeth that have matured before the time of fluoride absorption are not affected by fluorides.[1, 7, 68, 63]

At a stage more advanced than that causing the effect on forming teeth, a distinctive bilateral lameness and stiffness of gait may develop in cattle. Since commonly there is no reason for examination of a cow's teeth, the development of lameness in fluoride-affected cattle may be the first *noticed* symptom; although, if the lameness is due to fluorides, a subsequent check of the teeth should show a mottling and wear of teeth that were forming during the period of fluoride ingestion. Lameness may be accompanied by exostoses on the long bones, jawbone, and ribs. As further amounts of fluorides continue to be absorbed, the exostoses may be palpated and there may be an observable thickening of the long bones, mandibles, and ribs.[51, 63] It should be noted that dietary disturbances such as phosphorus or vitamin D deficiences, as well as mechanical injuries, also may result in lameness or stiffness. Therefore, one must look for characteristic exostoses on, or thickening of, the long bones as a confirming symptom of fluorosis.

The combined involvement of the teeth and the bones, as described, are unique lesions of fluorosis and should be the first for which a search is made. Many other symptoms or suspected symptoms of fluorosis have been reported in the literature, among them being dryness and stiffness of hide, poor condition of the hair coat, elongated hooves, diarrhea, impaired appetite, decreased weight gain, lowered milk yield, emaciation, cachexia, and anemia.[1, 3, 6, 7, 8, 30, 37, 38, 40, 51, 53, 58] None of these symptoms is unique to fluorosis, however, and the validity of any of them as indexes of fluorosis should be checked by correlation with tooth and bone involvement, as well as confirmation through laboratory analysis for fluorine in feed and drinking water, urine, and (where possible) bones.

Dryness and stiffness of hide and impaired appetite have been reported frequently as symptoms of fluorosis of a moderately severe type, and may be considered typical symptoms if the sensitive indexes of tooth and bone involvement are present. A change in characteristics of the hair coat can result from other dietary problems, such as a deficiency in or a severe excess of copper or cobalt.[44, 45]

Impairment of appetite may result through dietary deficiencies, such as lack of adequate digestible protein or deficiency in phosphorus, or because of diseases of the mouth or digestive system, although there is ample evidence that it is a symptom of advanced damaging fluorosis (possibly due to difficulty in foraging and mastication), when combined with the specific lesions of tooth and skeletal involvement.

Diarrhea occasionally has been mentioned in connection with severe fluorosis.[7, 55, 58] In field examinations where opportunity has been afforded to go into the matter further, however, it has been found that the diarrhea has resulted from other causes such as Johne's disease.[7] In those few cases where diarrhea has been observed in controlled feeding experiments, the amount of fluoride fed has been far in excess of amounts that could be considered borderline for cattle. Roholm,[58] in listing the symptoms recorded for 24 separate feeding experiments, mentions diarrhea as a symptom in only two experiments, and in both cases, the dosage of fluoride is so high that fluorosis could be considered acute rather than chronic. Diarrhea results from so many causes, such as Johne's disease, various parasites, copper or cobalt deficiencies, ingestion of some toxic weeds, or even change in pasture condition, that ordinarily it can not be considered a symptom of fluoride poisoning. There remains the possibility, however, that it may be a result of absorption of large amounts of fluoride, in the range that would produce acute poisoning. If such amounts of fluoride were endured for any period of time, however, tooth and bone involvement would be clearly evident.

Decreased weight gain, lowered milk yield, emaciation, cachexia, and anemia are symptoms of damaging fluorosis that may make themselves apparent at an advanced stage of fluorosis. They may result either from prolonged absorption of fluorides, with subsequent inability of the skeletal structure to act as a buffer

or storage reservoir, or from reduced food intake, due to the inability of the animal to forage and masticate, as a result of extreme lameness and severely worn teeth. It would not be expected, however, that any of these symptoms would develop, even over an extended period, at borderline levels of fluoride absorption that were just sufficient to mottle teeth or cause a slight wear in the teeth. It should be noted also that all of these symptoms might be caused by dietary abnormalities other than excessive fluoride.

Morbid Anatomical Findings.—Bones and teeth, where fluorine accumulates to the greatest degree in the body, exhibit characteristic changes with excessive fluoride absorption. Hypoplasia of the tooth enamel is observed. Bones develop a chalky white appearance, brittleness is increased, and, with increasing absorption, exostoses develop, resulting in increased diameter of the bones.[53]

Although Roholm[55] and others describe a number of pathological changes that take place as a result of the absorption of fluoride, the controversial nature of some of the findings and the fact that most of the changes occur only at a high level of fluoride absorption make them of little value in the evaluation of a situation where the amounts of fluoride ingested are low or marginal (*i.e.*, fluorine ingestion of 3 mg. per kilogram of body weight per day or less).

In severe cases of fluorosis, degeneration has been reported in the kidney and liver and changes in size in a number of the endocrine glands, particularly the thyroid, parathyroid, and suprarenal glands. Fluorides in moderate amounts do not cause any distinct changes in the gastrointestinal tract, although when amounts are large, inflammatory changes are noted in the stomach.[58]

Biochemical Findings.—The most sensitive and definite biochemical indication of *past* fluoride absorption is a large increase in the fluorine content of the bones and teeth.[8] The bones accumulate fluorine to a greater degree than the teeth. Because of this ability, bone analysis provides a valuable means of checking on the past absorption of fluoride. Also, properly interpreted, the fluorine content of the bones will provide indications as to whether or not the animal has taken in enough fluoride to cause damage.

A fluorine content between 60 and 900 parts per million (p.p.m.)* in bones from animals not unduly exposed to fluoride has been reported by Agate *et al.*,[1] Blakemore *et al.*,[7] Evans *et al.*, [14]Gaud *et al.*,[17] Hatfield *et al.*,[20] Kick *et al.*,[22] Meyn and Viehl,[28] Peirce (quoting Salinas Y Ferrer),[46] Phillips *et al.*,[51] Slagsvold,[58] and many others. It is also the opinion of Agate *et al.*,[1] Meyn and Viehl,[28] Mitchell,[40] Peirce,[45, 46] Phillips *et al.*,[51] and others that the normal fluorine level in bone can be exceeded by from seven to fifteen times without detrimental effect on the animal other than possible mottling or slight wear of teeth. Below are tabulated some of the normal bone contents that have been reported and some of the fluorine contents of bones of cattle and swine where no effect has been observed except possible wear or mottling of teeth.

It is concluded that, where bone analyses for fluorine are available, the following conclusions are reasonably well established:

1) Fluorine content of bone up to 600 p.p.m. will be found in normal animals that have never absorbed abnormal amounts of fluoride.

2) Fluorine content of bone as high as 3,000 p.p.m. will be encountered in animals that have not been affected by fluoride beyond a possible slight mottling or wearing of the teeth. Animals that are definitely damaged by fluoride and that have not been

Investigator	Fluorine in normal bone (p.p.m. dry fat-free basis)	Fluorine in bone of animal with no effect except teeth (p.p.m. dry fat-free basis)
Agate *et al.*[1]	60– 115	.....
	125– 465	.....
Bardelli and Menzani[3]	540– 720	1,000–1,320 (initial or limping)
Blakemore *et al.*[7]	300– 600	.....
Evans *et al.*[14]	177– 509	.....
Gaud *et al.*[17]	125– 240	.....
Hatfield *et al.*[20]	350 (ave.)	2,030–2,800 (no tooth damage)
Kick *et al.*[22]	300–1240	1,300–3,200 (no tooth damage)
Meyn and Viehl[28]	360– 480	.....
Peirce[46]	260– 570	5,200–7,900 (metacarpals)
Phillips *et al.*[51]	569– 601	5,354 (ave.-metacarpals)
Slagsvold[58]	... 580	.....

*In this paper, fluorine concentrations are reported on the following bases:
in forage and feeds—on a dry weight basis, as p.p.m.; in water—in p.p.m.; in urine—corrected to a specific gravity of 1.040, as p.p.m.; in bone—on a dry, fat-free basis.
(One part per million equals 0.0001 per cent.)

taken off their high fluoride diet should exhibit fluorine content of bone well in excess of 4,000 p.p.m.

Agate *et al.*[1] and Blakemore *et al.*[7] have used the fluorine content of urine as a partial measure of the *current* absorption of fluorine-containing materials. They consider that the urine of cattle ingesting normal amounts of fluoride will contain less than five p.p.m. of fluorine, and that values less than ten p.p.m. do not represent current absorption sufficient to cause any damage to the animal. In all cases where animals were definitely damaged, the urine level was well in excess of ten p.p.m. of fluorine. It should be noted that high fluorine content urine can result both from high current fluoride intake and from spillage from stored fluoride in the skeleton.

There is little indication that the fluorine content of the blood increases appreciably with large amounts of fluoride ingestion.[22] The fluorine level in milk is low, regardless of amounts ingested.[7, 51] Most of the internal organs have been shown to accumulate relatively small amounts of fluorine, with the exception of the thyroid[4, 55] and the kidney.[8]

Establishing a Borderline Fluoride Ingestion Level.—Although it has not been established that fluoride plays a necessary role in the metabolism of the cow, it is true that all cattle ingest small amounts of fluoride regularly with no ill effect, since all forage vegetation contains fluorine to some degree. This natural amount may vary from one or two p.p.m. in the case of some grasses, up to twenty or thirty p.p.m. in the case of legumes such as clover and alfalfa, growing in some soils that are rich in fluorine.

In order to determine whether or not a cow has been ingesting fluorine compounds in potentially damaging amounts, it is first necessary to have a clear idea of the maximum total amount of fluorine that a cow can absorb each day with no damage. If, after careful sampling and analysis, it can be demonstrated that the animals under suspicion in the field investigation have been regularly absorbing amounts in excess of this borderline limit, a basis exists for properly confirming the symptoms of fluorosis observed in the field. In determining the total amount of fluorine being ingested, the forage-sampling program must be comprehensive enough to take account of differences in the ability of various plant species to accumulate fluorine, seasonal differences in fluorine content of vegetation, and other factors that will inevitably be encountered in the sampling program. Single samplings or small groups of samplings can be very misleading.

It appears that there have been only two attempts to assess the literature critically in order to determine the amount of fluoride that will damage cattle. The Association of American Feed Control Officials, in establishing its tentative regulation TR-1 for fluorides,[2] must have examined the data available, and Dr. H. H. Mitchell, in his excellent monograph for the National Research Council,[40] has critically compared the available data in order to determine the borderline level and recommend safe tolerances for practical feeding.

In general, there are two sources of data on the toxicity of fluorides to domestic animals: reports of controlled feeding experiments and reports of field investigations of areas where fluoride toxicosis or suspected fluoride toxicoses have been discovered. Because of the very nature of field investigations of endemic fluorosis areas, the data obtained are often ill suited to help in determining the borderline between damaging and nondamaging levels of fluoride absorption. Samplings to determine amounts ingested have been fragmentary and, in many of the older investigations, the analytical methods used were less reliable than the analytical techniques developed in the past twenty years. On the other hand, the many field investigations have developed considerable information on the symptoms of fluorosis and, for this reason, some of the more recent investigations will be included in this critical survey of the literature.

The most reliable information on the borderline levels of fluoride intoxication can be derived from recent controlled feeding experiments. Among these, the most valuable are those that have been conducted since about 1930, because of the variability of analytical techniques in use prior to that time. The difficulties with the older analytical methods have been well summarized by McClure:[37]

"Disagreement among analysts reporting quantitative results for fluoride, wherever it occurs in very small amounts, may be attributed largely to difficulties involved in the analytical procedure. The notable chemical activity of fluorine, as well as the volatile nature of many of its compounds, has contributed to these difficulties. Despite the fact that numerous methods have been devised and have been used, at least by their originators, it is not established that any fluoride method now known is adequate for the quantitative analysis of plant and animal substances."

The above statement was printed in 1933. Although McClure goes on to discuss many of the older gravimetric and volumetric procedures, he was evidently unaware of the then recent development of the Willard and Winter

method,[47] which has since, with various modifications,[6, 54, 57] come to be almost universally used in this country because of its superiority in providing reliable results when fluorides are accompanied by interfering elements such as aluminum.* Later, however, McClure used the the Willard and Winter method.[36] The Willard and Winter method is also the official method of the Association of Official Agricultural Chemists. Therefore, although the present literature study has included many of the older controlled feeding experiments, reliance has been placed primarily on controlled feeding experiments that have been conducted since 1930 on cattle, sheep, and swine. Feeding experiments on small laboratory animals, although valuable for establishing the relative toxicities of various fluorine compounds, can not be used to predict the absolute levels that will be toxic to large animals.

Field Investigations. — Agate and coworkers,[1] in their investigations of fluorosis in the Fort William area in Scotland, found marked chronic fluorosis in cattle and sheep, with symptoms of excessive tooth wear, lowered gain in weight, loss in milk production, and general poor health. In the case of 2 sheep, the fluorine content of the mandibles was 5,600 to 7,700 p.p.m. A sample of herbage taken from the pasture on which these animals had been grazing analyzed 61 p.p.m. of fluorine. Another sheep, with the same symptoms, had been grazing on forage analyzing 44 p.p.m. The mandible of this animal contained 4,600 p.p.m. of fluorine. In an area of fifty square miles, the investigators took only thirty-three samples of forage. These ran up as high as 1,104 p.p.m. of fluorine, with the majority ranging between 20 and 100 p.p.m. The fact that the investigators were not able to measure the actual level of fluoride ingestion is clearly pointed out in their report: "In the circumstances prevailing in the Fort William area, it was impossible to attempt to assess the daily fluorine intake of the animals."

Bardelli and Menzani,[3] reporting in 1937 and 1938 on their investigations of chronic fluorosis surrounding an aluminum plant in Italy, found symptoms of lameness, exostoses on the long bones, dry hide, decreased weight gain, and lowered milk production. Analyses of forage in the vicinity of the factory showed from 460 to 720 p.p.m. of fluorine in the air-dried hay. Their analytical results must be viewed with considerable skepticism, since they report uncontaminated hay to contain 120 p.p.m. of fluorine, an unusually high figure.

Blakemore, Bosworth, and Green reported, in 1948,[7] on their investigations of fluorosis in cattle pastured in the vicinity of a number of brick factories in England. They found that the bones of affected animals contained from 6,000 to 16,000 p.p.m of fluorine and concluded: "For values on 'bone ash,' fluorine values of 500-1,000 p.p.m. are regarded as quite normal, but can be greatly exceeded before fluorosis is clinically detectable." They found lameness and skeletal changes to occur only where levels of fluorine in pasture exceeded 25 p.p.m. and found mild cases (tooth mottling) with pasture containing 14 to 16 p.p.m. of fluorine. These conclusions appear unwarranted in view of the fact that they took only fourteen samples of forage in the affected area of about ten square miles, and only single samples at each location.

Gaud *et al.*,[17] investigating endemic fluorosis in the "darmous"* area of North Africa, found that jawbones of affected sheep contained from 1,200 to 4,400 p.p.m. of fluorine, and calculated that they had been ingesting about 6 mg. of fluorine per kilogram of body weight per day, when the sources of water, mud, vegetation, and dust were taken into account. The only lesion mentioned was worn and mottled teeth.

Meyn and Viehl,[38] in 1941, investigated the occurrence of fluorosis in cattle on a baronial estate near Dresden, Germany, the estate being in the vicinity of a factory giving off fluorine compounds. Cattle and sheep on the estate exhibited a high degree of wear of the incisors, lameness, emaciation and cachexia, and a reduction in milk production. Various bones from affected cattle analyzed from 3,460 to 5,530 p.p.m. of fluorine, an increase of about ten times the normal amount. Various samples of air-dried feeds ranged from 67 to 234 p.p.m. of fluorine on a dry basis, and ensiled turnip leaves analyzed 75 p.p.m. of fluorine, dry basis. Based on a typical formula using a high proportion of ensiled turnip leaves, the daily ingestion of fluorine was approximately 3.4 mg. per kilogram of body weight.

Many of the water supplies in the Pretoria district of South Africa contain abnormal amounts of fluoride. Ockerse[46] found mottled enamel and excessive attrition of the teeth in cattle reared on a farm, the water supply of which had a fluorine content of 11.78 p.p.m.

Peirce,[49] in his literature survey, noted that, in the Argentine, cattle that drank water containing 10 p.p.m. of fluorine had sound molars.

*Researches currently being carried on at Oregon State College and Stanford Research Institute give some indication that the Willard and Winter analytical methods as presently used may result in incomplete recovery of fluoride from certain forages and roughages. If these preliminary findings are substantiated, it may develop that a somewhat higher toxic level of fluorine will be indicated, as well as a somewhat higher measured amount of fluorine in field studies.

*Term as used in this area synonymous to damaging fluorosis.

Slagsvold[28] found that hay from the vicinity of a Norwegian aluminum plant contained from 200 to 250 p.p.m. of fluorine. The analytical results, which were obtained by the Steiger colorimetric method, are open to question, since he reports uncontaminated hay as containing 50 p.p.m. of fluorine. He noted that sheep fed on the contaminated hay began to lose their appetite within three months, and that at the end of a year the sheep were anemic, stiff, and had developed exostoses.

Controlled Feeding Experiments.—In 1936, Elmslie reported on a small but extended feeding experiment concerning the feeding of 2 dairy cows with a ration containing rock phosphate in the mineral supplement.[12] Over a period of five years, with fluorine ingestion ranging between 1.5 and 2.0 mg. per kilogram of body weight per day, the cows behaved normally in all respects. The gains in weight were normal, milk and butterfat production was normal, and reproduction (5 calves for each cow) was normal. There was no anorexia or inanition, no delayed estrus, and no falling off in condition. The teeth of both animals became slightly abraded and slightly uneven.

Fargo and co-workers,[16] using rock phosphate, found that teeth became defective in brood sows when the feed contained 140 p.p.m. of fluorine. They found that in some manner green feed raised the tolerance of swine for fluoride, and concluded that swine have a higher tolerance for fluoride than cattle.

Hatfield, Shrewsbury, and Doyle[29] conducted a controlled feeding experiment with western lambs for a period of 170 days. At levels of ingestion of 1.5 and 3.0 mg. of fluorine (as rock phosphate) per kilogram of body weight per day, the sheep showed improved usage of feed and improved rate of growth. Fluorine consumption at the rate of 6 mg. per kilogram of body weight per day resulted in decreased grain consumption and rate of growth. Because of the shortness of the experimental period, this experiment is of little value in our present critical study of the literature to determine the borderline level for long-term ingestion of fluorine, except to illustrate the sensitivity of the fluorine content of bones as an index of fluorine absorption. In the relatively short period of the experiment, the fluorine content of the left humerus went from 350 p.p.m. (control) to 2,030 p.p.m., on a diet containing 1.5 mg. of fluorine per kilogram of body weight per day.

Kick and his colleagues[22] reported, in 1935, on a very comprehensive series of experiments concerned with the feeding of fluoride in different forms to swine. Unfortunately, for our present purpose, the relatively short periods of the experiments shed little light on the effects of longer-term fluoride ingestions. However, the level of fluorine in the bones again showed the sensitivity of this index, even over short periods. It was concluded by the authors that 175 p.p.m. of fluorine as rock phosphate or 100 p.p.m. of fluorine as sodium fluoride could be fed with no effect on daily gains in weight. In a period of 160 days, the feeding of 100 p.p.m. of fluorine as sodium fluoride raised the content of fluorine in the femur from 600 p.p.m. (in the control) to 3,200 p.p.m. The same amount of fluorine in the feed (100 p.p.m.) as rock phosphate increased the femur content from 300 p.p.m. in the control to 1,300 p.p.m.

Majumdar, Ray, and co-workers[30, 31] recently conducted a series of feeding experiments in India. Over a nine-month test on adult hills bulls, they found that 3 mg. of fluorine per kilogram of body weight per day (as sodium fluoride) caused severe symptoms of fluorosis and death in nine months, when fed on a low phosphorus diet. The same amount, fed on a diet containing adequate calcium and phosphorus, produced only mild symptoms, namely, wearing of the incisors and enlargement of the hoofs. The results of this experiment are of little value in determining the damaging level of fluorine because of the subnormalities introduced into the diets and the relatively short period of the test.

One of the most complete feeding experiments of recent years has been conducted by Peirce in Australia.[46] With rock phosphote as the source of fluoride, varying amounts were fed to yearling sheep over a period of three years. In other respects, the diet was normal, and all groups received the same amount of phosphorus daily. At an average level of 2.3 mg. of fluorine per kilogram of body weight per day, the sheep grew normally and remained in good health throughout the experiment. The incisors and molars were pitted and the molars selectively abraded, which was also the case even at a level of 1.2 mg. per kilogram of body weight per day. Amounts of fluorine greater than 2.3 mg. per kilogram affected the animals adversely, causing a decline in food consumption and weight increase and impairment in general health. The metacarpals of the sheep fed 1.2 mg. of fluorine per kilogram analyzed 5,900 p.p.m. of fluorine, while the metacarpals of the 2.3 mg. of fluorine per kilogram group analyzed 7,000 p.p.m. of fluorine. These figures show more than a tenfold increase from the average of 410 p.p.m. in the metacarpals of the control group.

Phillips, Hart, and Bohstedt[21] conducted an extensive test on dairy cows at the University of Wisconsin, Madison, feeding varying amounts of fluoride, with rock phosphate as the fluorine source. The experiments continued over four years and constitute a valuable contribution to the problem of determining the borderline between the amounts of fluoride that are damag-

ing and those not damaging when absorbed over a long period. With a total diet containing an average of 82 p.p.m. of fluorine, growth over the entire period was normal, with a slight but statistically insignificant drop in milk production. Some exostoses were produced and the molars were abraded. Breeding was unimpaired. The average fluorine content of the metacarpals of this borderline group was 5,354 p.p.m. as compared with 584 p.p.m. in the metacarpal of the control. The authors concluded that the critical margin of fluorine tolerance in dairy cows was between 2 and 3 mg. per kilogram of body weight per day.

Reed and Huffman[50] conducted a five-year, rock phosphate-feeding investigation with dairy cattle, in which they found that 158 p.p.m. of fluorine in the diet caused badly worn teeth, exostotic bones, ankylosis, lowered appetite, and retarded growth. Milk production was also seriously affected. Unfortunately (for the present purpose of establishing a borderline level) smaller amounts of fluoride were not fed.

Tolle and Maynard[60] at Cornell University, Ithaca, N. Y., conducted a four-month test on pigs. An amount of 276 p.p.m. of fluorine in the diet (as rock phosphate) produced no bone changes or tooth mottling (deciduous teeth). The length of the experiment was not sufficient to provide any information of value for the present question.

In evaluating the information obtained in the investigations outlined above (and summarized in table 1) primary reliance has been placed upon the controlled feeding experiments of Elmslie,[12] Peirce,[48] and Phillips *et al.*[51] These investigations were of long duration, and they were made recently enough so that advantage could be taken of the improved analytical techniques that have been developed. Also, all three investigations were carried out on ruminants. The tabulation below shows the results as obtained by these three investigators.

Investigator	Years of expt.	Subjects	Material fed	Borderline fluorine ingestion (mg./kg. b.w./day)
Elmslie[12]	5	Milk cows	Rock phos.	1.5-2.0
Peirce[48]	3	Sheep	Rock phos.	1.2-2-3
Phillips[51]	4	Milk cows	Rock phos.	2.0-3.0

In the above tabulation, the fluoride ingestion of the animal in milligrams per kilogram of body weight per day is the borderline amount in that no effect on the experimental animals was noted except slight tooth mottling or wearing, and is the amount that the investigators concluded was the maximum safe limit that could be fed in that form.

If the mean of the extremes in the above tabulation is taken as the effective borderline level, this results in a figure of 2.1 mg. of fluorine per kilogram of body weight per day. These figures are in reasonably good agreement with Mitchell[40] and the Association of American Feed Control Officials.[2] Mitchell would allow as high as about 3 mg., based on his own experiments and a study of the work of others. The tentative regulations of the Association of American Feed Control Officials will permit an ingestion up to a maximum of about 2 mg. of fluorine per kilogram of body weight per day, with a liberal allowance presumably having been made for the uncertainties of practical animal husbandry.[2, 40, 42]

The conclusions reached in the preceding paragraphs on the borderline amounts of fluoride permissible for cattle are based on the fluorine in rock phosphate. In the following section of this report, it is demonstrated that the fluorine in sodium fluoride (the most soluble of the fluoride salts used) is absorbed twice as readily as the fluorine in rock phosphate, at the levels of ingestion with which we are concerned. It may therefore be concluded that, for a constant ingestion over an extended period, the borderline ingestion level of fluorine for the more soluble compounds (such as sodium fluoride) is 1 mg. of fluorine per kilogram of body weight per day.

The Relative Toxicities of Inorganic Fluorides.—In field investigations having to do with possible fluoride poisoning of cattle, the fluorine salt contaminating the forage may be more soluble (and consequently more toxic) than rock phosphate. In the case of relatively insoluble compounds, such as rock phosphate, the larger particles are apparently only partially dissolved in the animal and, consequently, only part of the fluoride is effective. The solubilities of some of the fluorine-containing compounds are as follows:[53]

Sodium fluoride, NaF
4.2100 Gm. per 100 cc. at 25 C.

Sodium silico-fluoride, Na_2SiF_6
0.7590 Gm. per 100 cc. at 25 C.
Cryolite, Na_3AlF_6
0.0630 Gm. per 100 cc. at 25 C.
Calcium fluoride, CaF_2
0.0017 Gm. per 100 cc. at 25 C.

In the preceding section of this study, primary reliance was placed upon controlled experiments feeding rock phosphate to cattle as a means of determining a safe borderline ingestion level. In order that this may be translated into safe levels for other more soluble fluorine salts, it is necessary to study the comparative toxicities as established by small animal feeding experiments.

There is general agreement among investigators that calcium fluoride is the least toxic of the inorganic fluorine compounds tested and that sodium fluoride and sodium silico-fluoride are the most toxic. From most of the data in the literature, it would appear that cryolite is only slightly more toxic than calcium fluoride. Phosphate rock, containing about 3½ per cent fluorine, occupies an intermediate position between the most and least toxic materials mentioned previously. It has also been demonstrated by several investigators that, at low levels of fluoride ingestion, the differences in toxicities, which are due to the relative solubilities, disappear, and all of the previously mentioned compounds exhibit the same toxicity. This is because all of the salts are completely soluble at these levels.

The Association of American Feed Control Officials evidently considers all fluorine compounds, or at least those commonly used in mineral supplements, to be of equal toxicity, since it does not apply different tolerances to different materials.[2]

Evans *et al.*,[15] in experiments with rats, found that at low levels of fluorine ingestion (4 p.p.m. in the drinking water) the toxicities of sodium fluoride and cryolite were equal, as measured by fluorine storage in the bones. Using inhibition of growth as a criterion, they found that when 600 p.p.m. of fluorine were included in the diet, sodium fluoride was twice as toxic as cryolite. They concluded that at higher concentrations the toxicity was roughly proportional to the soluble fluorine content.

Haman *et al.*[17] rated several fluorine compounds in the following order of decreasing toxicity, based on experiments with hens: fluosilicates, sodium fluoride, phosphate rock, and calcium fluoride.

Kick *et al.*[23] found that, at levels of from 100 to 300 p.p.m. of fluorine, sodium fluoride was no more toxic than rock phosphate, based on storage of fluorine in the bones. At higher levels, they found that calcium fluoride was the least toxic, sodium fluoride and sodium fluosilicate the most toxic, with triple superphosphate, phosphate rock, and phosphatic limestone occupying intermediate positions. These tests were conducted on swine and ran for periods from 140 days to two years.

Using rats through five generations, Lamb *et al.*[24] found the addition of 350 p.p.m. of fluorine as rock phosphate (40 mg./kg. of body weight/day) produced the same results as 190 p.p.m. of fluorine as sodium fluoride (20 mg./kg. of body weight/day), namely, dental fluorosis and slight inhibition of growth. The toxicity of calcium fluoride appeared to be about the same as that of rock phosphate.

At a level of 2,900 p.p.m. of fluorine per day (about 50 mg./kg. of body weight/day), both sodium fluoride and cryolite resulted in the same storage in the bones, according to an experiment run by Largent *et al.*[25] for fifty days.

On one human subject, Largent and Heyroth[21] found that, at low levels of ingestion (6 to 36 mg. of fluorine per day), the fluoride ions derived from cryolite, bone meal, and calcium fluoride were all absorbed to the same extent. Fluoride in solution, however, was stored about 40 per cent more readily than in the case of fluorine-containing compounds in the dry state.

Lawrenz and co-workers[27] found that when fluoride as cryolite was fed at a rate equivalent to about 10 p.p.m. in solid food, it was retained in the bones and tissues to an extent of about 20 per cent less than when administered in the drinking water. They also found[28] that fluorine in cryolite is no more toxic and is retained in the body no more than fluorine in calcium fluoride when both are fed in aqueous solution at the rate of 0.58 mg. per kilogram of body weight per day. These experiments were all performed on rats.

Machle and Largent[29] concluded that the absorption of fluorine was dependent upon the aqueous solubility of the fluorine salt and its physical state at the time of ingestion.

Marcovitch[30] found that rats stored fluorine nearly twice as fast in the body when it was fed as dissolved fluorine in the drinking water than when fed as cryolite in the food (4 p.p.m. in the food).

Peirce[46] quotes Velu (1932) to the effect that a dose of 0.5 mg. of fluorine per kilogram of body weight per day in the drinking water of sheep produced the same effect as 20 mg. per kilogram of body weight per day when fed in the form of rock phosphate mixed with the food.

Schulz,[55] based on experiments with rats, found that sodium fluoride ranged from twice as toxic to eight times as toxic as calcium fluoride. He found the absorbed fluorine in rock phosphate to be almost as toxic as equivalent fluorine from sodium fluoride. The toxicity of

superphosphate was about the same as calcium fluoride, and the fluorine in phosphatic limestone was less toxic than that in rock phosphate.

Smith and Leverton[62] found that 226 p.p.m. of fluorine as sodium fluoride was harmful to the growth of rats, but it took ten times as much fluorine as cryolite or twenty times as much as calcium fluoride to achieve the same effect. Based on ability to kill rats, they found that cryolite was twice as toxic as calcium fluoride and that sodium fluoride was sixty times as toxic as calcium fluoride. At fairly low levels of fluorine ingestion (56 p.p.m. of fluorine as CaF_2), sodium fluoride was twice as toxic as cryolite and four times as toxic as calcium fluoride, based on the effect on teeth. As the concentration of fluorine in the ration increased, the disparity in toxicities grew greater. The same effect on teeth is produced by 462 p.p.m. of fluorine as calcium fluoride, 113 p.p.m. as cryolite, and 56½ p.p.m. as sodium fluoride. This means that (based on tooth effect) at these levels, sodium fluoride is twice as toxic as cryolite and eight times as toxic as calcium fluoride.

Smith *et al.*[62] noted that at very low levels in rat experiments the difference in solubilities of the various inorganic fluorides no longer played any part and that all of the compounds mentioned previously had the same toxic effect (at 14 p.p.m. of fluorine in diet).

The information outlined above is summarized in table 2. Based upon a study of the available data, we have concluded that, at the level of fluoride ingestion that is safe for cattle, the fluoride in sodium fluoride is no more than twice as toxic as the fluoride in rock phosphate. At low or normal levels of ingestion, there is probably little difference in toxicity between sodium fluoride and rock phosphate. At a level of 2 mg. of fluorine per kilogram of body weight per day, which is borderline for cattle as far as rock phosphate is concerned, it can conservatively be concluded that the borderline with respect to sodium fluoride is no less than 1 mg. of fluorine per kilogram of body weight per day. Since sodium fluoride is soluble enough so that all of its fluorine is reactive and toxic, the level of 1 mg. per kilogram of body weight per day represents a standard of maximum severity at the levels of ingestion with which we are concerned.

SUMMARY

Occasional cases arise where it is suspected that cattle have been damaged through ingestion of abnormal amounts of fluoride that have been deposited on or assimilated by forage or as a result of emission of fluorine compounds to the air by industrial or other operations.

The field investigator should search for the unique lesions of fluorosis and obtain supporting laboratory data through analyses of forage, other feeds and water, urine, and bone for fluorine content.

Since there is usually no reason for examining cows' teeth, the first noticed symptoms of fluorosis in cattle may be bilateral lameness and stiffness, often accompanied by palpable exostoses on the long bones, lower jawbone, and ribs. However, prior

TABLE 2—Relative Toxicities of Unit Weights of Fluorine in Various Inorganic Fluorine-Containing Compounds

Ref.	Animals	F level in feed, p.p.m.	Mg./kg. b.w./day	NaF	Na_2SiF_6	Phos. lime	Phos. roc'k	Triple sup. phos.	Cryolite	CaF_2	Criterion
2	cattle	100	.....	.....	.....	1	1	1	.....	.....	Health
15	rats	4(H_2O)	.....	1	.....	.....	.....	.....	1	.....	F-bones
15	rats	600 (fed)	.....	2	.....	.....	.....	.....	1	.....	growth
19	hens	.....	.....	2nd	1st	.....	3rd	.....	.....	4th	.....
22	swine	.....	.....	1st	1st	3rd	2nd	2nd	.....	5th	.....
	swine	100-300	.....	1	.....	.....	1	.....	.....	.....	F-bone
24	rats	350 (as r.phos.)	40.00	2	.....	.....	1	.....	.....	1	growth
25	rabbits	2,900	50.00	1	.....	.....	.....	.....	1	.....	F-bones
26	man	6-36 mg./day	(F in aqueous solution stored 1.4 times as readily)						1	1	.....
27	rats	10	(F in aqueous solution stored 1.25 times as readily)						.....	.....	F-bones
28	rats	.....	0.58 (solution)						1	1	F-bones
33	rats	4	(F in aqueous solution stored 2 times as fast as F from dry cryolite)						.....	.....	F-bones
48	sheep	.....	(0.5 mg. F/kg. diss. in water = 20 mg. F/kg. as dry rock phos.)								
55	.....	.....	.....	2	.....	<2	sl.<2	1	.....	1	.....
55	.....	.....	.....	8	.....	<8	sl.<8	1	.....	1	.....
62	rats	4,520 (as CaF)	.....	20	.....	.....	.....	.....	2	1	Growth
62	rats	54,240 (as CaF)	.....	60	60	.....	.....	.....	2	1	Death
62	rats	56 (as CaF)	.....	4	4	.....	.....	.....	2	1	Teeth
62	rats	113 (as CaF)	.....	4	4	.....	.....	.....	2	1	Teeth
62	rats	462 (as CaF)	.....	8	8	.....	.....	.....	4	1	Teeth
62	rats	4,520 (as CaF)	.....	40	40	.....	.....	.....	10	1	Teeth
62	rats	9,040 (as CaF)	.....	40	40	.....	.....	.....	4	1	Teeth
63	rats	14 (all)	.....	1	1	.....	.....	.....	1	1	Teeth

Note: Least toxic material rated as 1, with other numbers showing number of times more toxic the fluorine is in the other materials, with exception of references 19 and 22, where most toxic material is listed "1st," next most toxic as "2nd," et cetera, on qualitative basis.

to these first-observed symptoms, the incisor teeth will exhibit ineradicable mottling and abnormal wearing, in those cases where the teeth were formed during the period of abnormal fluoride ingestion, and this tooth effect must be considered the first and most delicate indication of abnormal fluoride absorption. The involvement of teeth and bones are the only unique and easily recognized clinical lesions of fluorosis. In their absence, the investigator must seriously question whether fluorosis does in fact exist.

It should be stressed that at relatively low levels of fluoride ingestion, tooth effects may be of interest only as an indication that excessive fluoride absorption has occurred. The authors have seen many cattle, both beef and dairy, where mottled and worn teeth have not affected the value of the animals.[68]

In pronounced or advanced cases of fluorosis, cattle may exhibit dryness and stiffness of the hide, poor condition of the coat, diarrhea, impaired appetite, decreased weight gain, lowered milk yield, emaciation, cachexia, and anemia. These symptoms are not unique to fluorosis, and should always be checked by comparing with bone and tooth involvement and through analysis of forage, other feeds, water, urine, and bones.

When available, bone analyses for fluorine provide valuable laboratory support for clinical diagnosis. Bones from cattle not exposed to abnormal amounts of fluoride will normally contain less than 600 parts per million (p.p.m.) of fluorine. Bones from animals that have been damaged by fluorosis will analyze more than 3,000 to 4,000 p.p.m. of fluorine.

The urine analysis provides a method of determining whether or not cattle are presently absorbing abnormal amounts of fluoride and the possibility of the amounts absorbed being damaging. Cattle grazing on uncontaminated pastures will yield urine usually analyzing less than 5 p.p.m. of fluorine. No damage from fluorosis has been found unless the urine continuously analyzes well in excess of 10 p.p.m.

If facilities exist for adequately sampling the pasturage in a suspected area, an estimate can be made as to whether or not the amount of fluorine in the forage is in the damaging range for grazing animals. A safe borderline level for the most toxic of the commonly used fluoride salts (sodium fluoride) is 1 mg. of fluorine per kilogram of body weight per day.

It should be noted that because of the differing ability of various forages to accumulate fluoride, and because of seasonal and other variables, a comprehensive and long-term sampling and analytical program is necessary to determine total amounts of fluorides ingested. Single samplings or small groups of samplings can be very misleading.

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The Effect upon Cattle of Arizona Waters of High Fluoride Content

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IN RECENT years, there have occurred, both in this country and abroad, instances of damage or suspected damage to cattle as a result of the emission of fluorine compounds into the air from various industrial operations. The fluorides,* carried by the winds, settle upon or are absorbed by the forage in the near vicinity of the source and, consequently, may be ingested by grazing cattle.

Field investigations instigated to determine the degree and extent of involvement due to fluoride necessitate both the examination of the cattle (using primarily the unique symptoms of tooth mottling and enlargement of the long bones and ribs) and laboratory findings to support the diagnosis. The laboratory evidence usually is found through fluorine analyses of forages and feeds, water, urine, and bones. In properly interpreting the analytical data, one must have a clear idea of the normal fluorine contents of various forages and feeds, water, cattle urine, and bones, as well as knowledge as to what fluorine levels in these materials can be considered safe over long periods of continuous ingestion of fluorides.

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*The element fluorine, because of its extreme chemical activity, never occurs in the free state in nature, but rather always in combination with other elements. Compounds containing fluorine are commonly called fluorides. Therefore, in this paper, the word fluorine refers to the fluorine content of the particular fluoride in question.

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Much of the information available concerning the amounts of fluorine absorption that can be considered safe for cattle is derived from various long-term feeding experiments on ruminants (cattle and sheep) wherein rock phosphate was the source of added fluorine. The longest and most recent of these experiments have been made by Elmslie,[3] Peirce,[9] and Phillips *et al.*[10] A critical study by the authors of their experiments and others[11] indicates that when the source of fluorine is phosphate rock, cattle can ingest fluorides to the extent of 2 mg. of fluorine per kilogram of body weight per day over a period as long as five years, with no demonstrable effects noted other than a possible slight mottling and slight wearing of the teeth.

Fluoride emanations from industrial operations may be of a more soluble nature than fluorides in rock phosphate and, therefore, more readily absorbed in the animal system. The relatively soluble fluorine salt, sodium fluoride, has been compared with rock phosphate and cryolite with respect to toxicity in a number of feeding experiments using rats and swine. The literature offers no instances, however, of long-term feeding experiments with cattle where the source of fluorine was sodium fluoride (although several are now underway that should provide definitive answers in from three to five years). In order to convert the results of the feeding experiments using rock phosphate on cattle to terms of the effect of sodium fluoride on cattle, the authors recently evaluated[11] the work of Evans and Phillips,[4] Kick *et al.*,[7] Lamb *et al.*,[8] Schulz,[12] Smith and Leverton,[14] and others. It was determined that, at low levels of feeding of fluoride, there was no difference between the toxicity of absorbed fluoride from sodium fluoride and the absorbed fluoride from rock phosphate. At levels of fluorine which are borderline for cattle so far as rock phosphate is concerned, the fluoride in sodium fluoride may be absorbed in twice the quantities as the fluoride in rock phosphate. This is interpreted to mean that, if the fluoride

is more soluble in water (like sodium fluoride), the borderline between nondamaging and damaging amounts for cattle will be 1 mg. of fluorine per kilogram of body weight per day, when ingested over a long period, with no ill effects, although there may be slight mottling and slight wear of the teeth.

The use of urine analysis for fluorine as a means of confirming the current absorption of fluorides has been largely developed by Dr. H. H. Green and co-workers in England. They have found, based on many thousands of analyses,[2, 6] that fluorine contents up to five parts per million* (p.p.m.) are to be found normally, where no abnormal absorption of fluorides occurs. Damage to animals (even slight damage) occurs only when an average fluorine content of the urine of the herd is consistently above 10 p.p.m., with severe fluorosis evident only when the urine averages are well over 16 p.p.m. of fluorine.

The accumulative capacity of the bones for fluorine provides a means of determining the past exposure of animals. The authors, after a study[11] of the work of Blakemore *et al.*,[3] Hatfield *et al.*,[6] Kick *et al.*,[7] Peirce,[9] Phillips *et al.*,[10] and others, have concluded that animal bones containing 600 p.p.m. of fluorine or less are normal in the sense that they may be found in cattle that have never been exposed to fluorides other than those normally occurring in all vegetation. They have further concluded that bone contents of less than 3,000 to 4,000 p.p.m. of fluorine will be considered as not sufficient to confirm fluoride effects on cattle, with the possible exception of mottled or slightly worn teeth in the case of animals whose teeth were forming at the time of absorption of fluorides.

In an attempt to obtain direct analytical confirmation of the field study evidence and feeding experiment evidence offered in the literature, a study has been made of cattle on the northern outskirts of Phoenix, Ariz. In this area, many of the wells produce water high in fluorine content, to the extent that many children using such water at the time second teeth are forming develop severely mottled enamel. One of the first investigations of the effect of fluoride on teeth was carried out in these areas by Dr. Margaret Cammack Smith and co-workers[12] in the years just prior to 1936.

This report covers findings made on two farms in this area (Township 2N, Range 3E), where the high fluorine content of the sole source of drinking water for the cattle provides an ideal "natural controlled feeding experiment." A report is also given on another farm where fluorosis in cattle was experienced some years ago.

*In this paper, all fluorine values are reported as follows:
In forages and feed.....on a dry weight basis, as p.p.m.:
In water .....in p.p.m.:
In urine .....corrected to 1.040 specific gravity, as p.p.m.:
In bone.....on a dry, fat-free basis, as p.p.m.

THE MILKY WAY HEREFORD RANCH

The Milky Way Hereford Ranch maintains a Hereford breeding operation on the Home Farm on Camelback Road, Phoenix, and on the Scottsdale Farm 10 miles east of Phoenix. In practice, the cattle are fitted on the Scottsdale Farm, with the Home Farm being used as headquarters for the show string and for breeding and sales purposes. The Milky Way Hereford Ranch has been extraordinarily successful, ranking as one of the top Hereford breeding ranches in the United States.

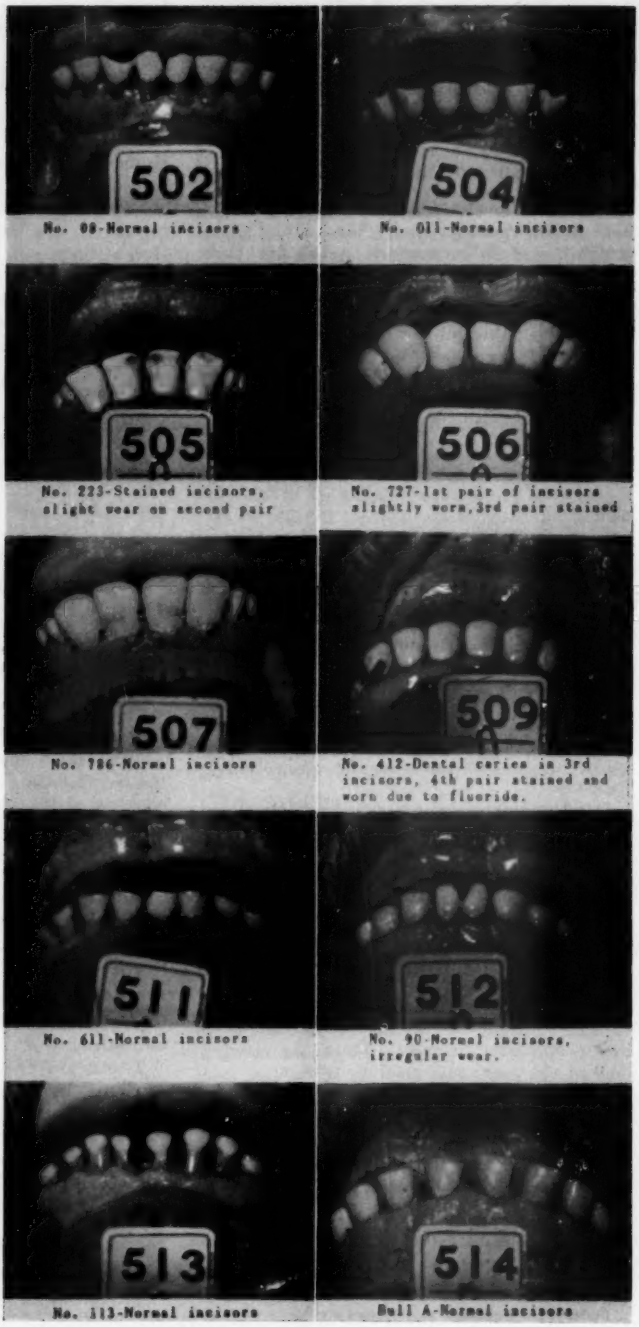
Fluorosis in their cattle has never been recognized as a problem or even suspected on the Milky Way Hereford Ranch. An examination of some selected stock, however, did show that 3 animals had some slight mottle and slight wear of the incisor teeth due to fluoride. The field notes of the examination of these animals, together with the fluorine analyses of the urine samples, are given in table 1. Only those animals that had spent at least half of their time on the Home Farm (where fluorides were highest) were examined. Some of the teeth photographs are shown in fig. 1.

It is apparent that under the usual practice of the Milky Way Hereford Ranch, where cattle spend most of their time on the Scottsdale Farm and where the fluorine content of the water is low, fluorosis would not be a problem. In the case of those animals that have spent at least half of their time on the Home Farm, where the fluorine content of the water and vegetation is high, there are indications that in some cases a marginal condition is approached. Some slight effect on teeth is noted, although there is no manifestation of bone involvement and no effect on the value of the animals.

There are two wells on the Home Farm. The well used as the only source of drinking water for the cattle has an average fluorine analysis of 16 p.p.m.** A sample of

**For comparative purposes, it may be noted that the drinking water of most communities in the United States contains much less than 1.0 p.p.m. of fluorine. In many cases, it will be as low as 0.1 p.p.m. or lower. A number of cities are now adding 1.0 p.p.m. of fluorine to the drinking water, since it has been found that this amount is beneficial to the teeth of children, sharply reducing the incidence of caries. Water contents of 1.5 to 2.0 p.p.m. and above will cause mottling of children's teeth.

Fig. 1—Teeth of Milky Way Hereford Ranch herd, Phoenix, Ariz.



water from the well used for irrigation analyzed 6.3 p.p.m. of fluorine. The hay and forage exhibited comparatively high fluorine contents. Samples of mixed forages from the Home Farm ranged from 15 to 25 p.p.m., while a composite sample of three lots of hay analyzed 6 p.p.m.

The only well on the Scottsdale Farm, used both for watering the cattle and irrigation, analyzed 1 p.p.m. of fluorine. Mixed pasture forage analyzed 11 p.p.m. and the hay contained 1 p.p.m.

The feeding formula of Milky Way calls for 10 per cent of hay and 90 per cent of pasture forage. Using this formula and the assumption that a 1,000-lb. animal will consume 21 lb. of feed and drink 10 gal. of water per day, the amount of daily fluoride intake of the cattle can be calculated.

While on the Home Farm, using fluorine values of 16 p.p.m. for drinking water, 20 p.p.m. for forage, and 6 p.p.m. for hay, a 1,000-lb. (453 kg.) animal will ingest 780 mg. of fluorine per day, equivalent to 1.7 mg. of fluorine per kilogram of body weight per day.

The same animal on the Scottsdale Farm, assuming drinking water at 1 p.p.m. of fluorine, forage at 11 p.p.m., and hay at 1 p.p.m., will ingest 135 mg. of fluorine per day, equivalent to 0.3 mg. of fluorine per kilogram of body weight per day.

The clinical examinations of animals that had spent at least half their time on the Home Ranch, where the fluoride intake is the higher, indicated that over a five-year period the maximum effect that occurred was a slight mottling and wearing of the incisor teeth. These animals, using the fluoride ingestions calculated above, averaged a fluoride intake of 1.0 mg. of fluorine per kilogram of body weight per day, on the basis that they spent half their time on each farm. This is in agreement with the conclusions drawn from the literature,[11] that cattle can ingest 1 mg. of soluble fluo-

TABLE I—The Milky Way Hereford Ranch

Ear tag or tattoo (No.)	Teeth photo (No.)	Urine sample (No.)	Description of animal	p.p.m. of fluorine in urine
08	502	.....	Born 1/5/40. On farm since 1945, entirely on Home Farm. Good show animal. Incisors and molars normal for age.	...
847	503	.....	Born 11/17/47. On Home Farm last nine months, previously on Scottsdale Farm. One pair incisors normal.	...
011	504	.....	Born 4/3/40. On Home Farm majority of time since 1945, incl. all time in past 2½ years. Enlarged hock, grass stain at base of incisors. Incisors and molars normal.	...
223	505	PU-2	Born 3/28/47. Purchased as yearling. Home Farm last 6 months, prior at Scottsdale. Two pairs incisors, both pairs stained, 2nd pair slight wear, texture normal. Molars normal. Possible marginal fluorosis damage to teeth.	21.1
727	506	PU-3.....	Born 1/9/47. Home Farm past year, half time before that since birth. 1st pair incisors slightly worn and chalky, 2nd pair normal, 3rd pair stained. Molars normal.	22.6
786	507	PU-4	Born 3/3/47. Home Farm past 9 months, half time before that. Two pairs incisors normal, molars normal.	8.4
707	508	PU-5	Born 1/12/47. Home Farm past year. Two pair incisors normal, 3rd pair just coming in. Molars normal.	12.7
412	509	PU-6	Born 1/12/44. Home Farm half time since 1945. Good breeding cow, very good general condition. 1st and 2nd pair incisors normal, 3rd pair dental caries, 4th pair stained and marked wear. Definite fluorine damage to teeth.	11.9
73	510	.....	Born 11/12/46. 1/3 time on Home Farm. On show string. Three pairs incisors normal, molars normal.	...
611	511	.....	Born 5/22/36. Home Farm ½ time since 1945. Incisors normal wear for age. Stiff.	...
90	512	PU-7	Born 5/29/39. More than half time on Home Farm, including 4/5 of last year. Irregular wear on center pair incisors but all incisors and molars normal.	22.6
113	513	.....	Born 2/26/33. Half time on Home Farm since 1945. Incisors and molars normal. Molars uneven, 1 and 2 worn even, not due to fluorine.	...
713	...	.....	Born 2/25/37. Incisors and molars normal.	...
Bull A	514	.....	Prince Domino A. 9 years old. On Home Farm full time since 1945. Incisors and molars normal	...
				16.5 (ave.)

rine per kilogram of body weight per day over a long period of time with no ill effect, except possible slight mottling and wear of the teeth.

The urine analyses for fluorine on this group of cattle are mostly in the range where the English investigators find the possibility of fluoride damage.[1, 2] It has been the experience of the authors that individual analyses can not be strictly interpreted because of the differing capacities of individual animals to absorb fluorides and that attention should be given only to herd averages. In the case of those animals from the Milky Way herd that were sampled, the average fluorine content of the urine was 16.5 p.p.m. This is somewhat higher than the borderline of 10 p.p.m. established by Dr. Green and must be attributed to the fact that when the samples were taken the animals were on the "high fluoride" farm, ingesting 1.7 mg. of fluorine per kilogram of body weight per day, which would undoubtedly be damaging over a long period if they did not eventually switch to the lower fluoride diet on the Scottsdale Farm.

In order to obtain some confirmation concerning the conclusion that damage to a cow would not occur until the fluorine content of the bones exceeded 3,000 to 4,000 p.p.m., a cow was obtained from the Milky Way Hereford Ranch and slaughtered. This cow (No. 122) was 3½ years old and a former grand champion. At the time of her slaughter, she weighed 1,240 lb. and was in excellent health. Her incisors and molars were normal, with no sign of stain or abnormal wear. She had been on the Home Farm, with high fluorine ingestion of 1.7 mg. of fluorine per kilogram of body weight per day, continuously for the previous two years.

A sample of her urine taken just prior to slaughter analyzed 11.2 p.p.m. of fluorine. The fluorine content of the meta-

TABLE 2—The Homrighausen Dairy Farm

Ear tag or tattoo (No.)	Teeth photo (No.)	Urine sample (No.)	Description of animal	Pounds fat prod. (365 days)	p.p.m. of fluorine in urine
86-4549	...	.....	8 yr. old, born on place. 3 pairs incisors normal, 4th pair slight question.	550	...
39134	...	.....	13 yr., born on place. Teeth normal.	580	...
10187	516	.....	Born 12/2/41 on place. Teeth normal.	480 (280 days)	...
Iris	517	.....	Born 1/14/42 on place. Teeth normal.	500	...
W559858	518	.....	On place 2 months. 13 yr. old. Teeth excellent.	500	...
34528	...	.....	Born 2/20/43 on place. Teeth normal.	566	...
Z106	159	.....	Born 9/25/45 on place. Teeth normal.	525	...
73151	...	.....	Born 3/18/40 on place. Teeth normal.	550–600	...
34534	520	.....	Born 2/26/44 on place. 1st 3 pairs incisors normal. 4th pair very slight stain.	500	...
34535	...	.....	Born 11/7/44 on place. Teeth normal.	485	...
Patty E	521	PU–14	2 yr. old, born on place. 1 pair incisors normal.	...	6.3
256 Vesta G	...	PU–17	3 yr. old, born on place. Teeth normal.	440	8.5
Z202 Linda	522	PU–15	3 yr. old, born on place. 1st two pairs incisors normal. 3rd pair slight stain, almost mottled.	305	9.1
86-3857 Vesta B	...	.....	Born 3/25/40 on place. Teeth normal.	500	...
26191 Whitey C	525	PU–18	Born 2/9/41 on place, teeth normal. Lame.	550	17.1
10181	523	.....	3 yr. old, born on place. Teeth normal.	550	...
Z291	524	.....	3 yr. old, born on place. Teeth normal. Slight stain on 3rd pair incisors.	430	...
Z205	...	.....	3 yr. old, born on place. Teeth normal.	420	...
Z258	526	PU–16	Born 4/9/47 on place. Teeth normal, gingivitis.	305	5.9
39136	...	PU–21	Born 1/15/37 on place. Teeth normal. Lame, no exostoses.	560	8.9
					9.3 (ave.)

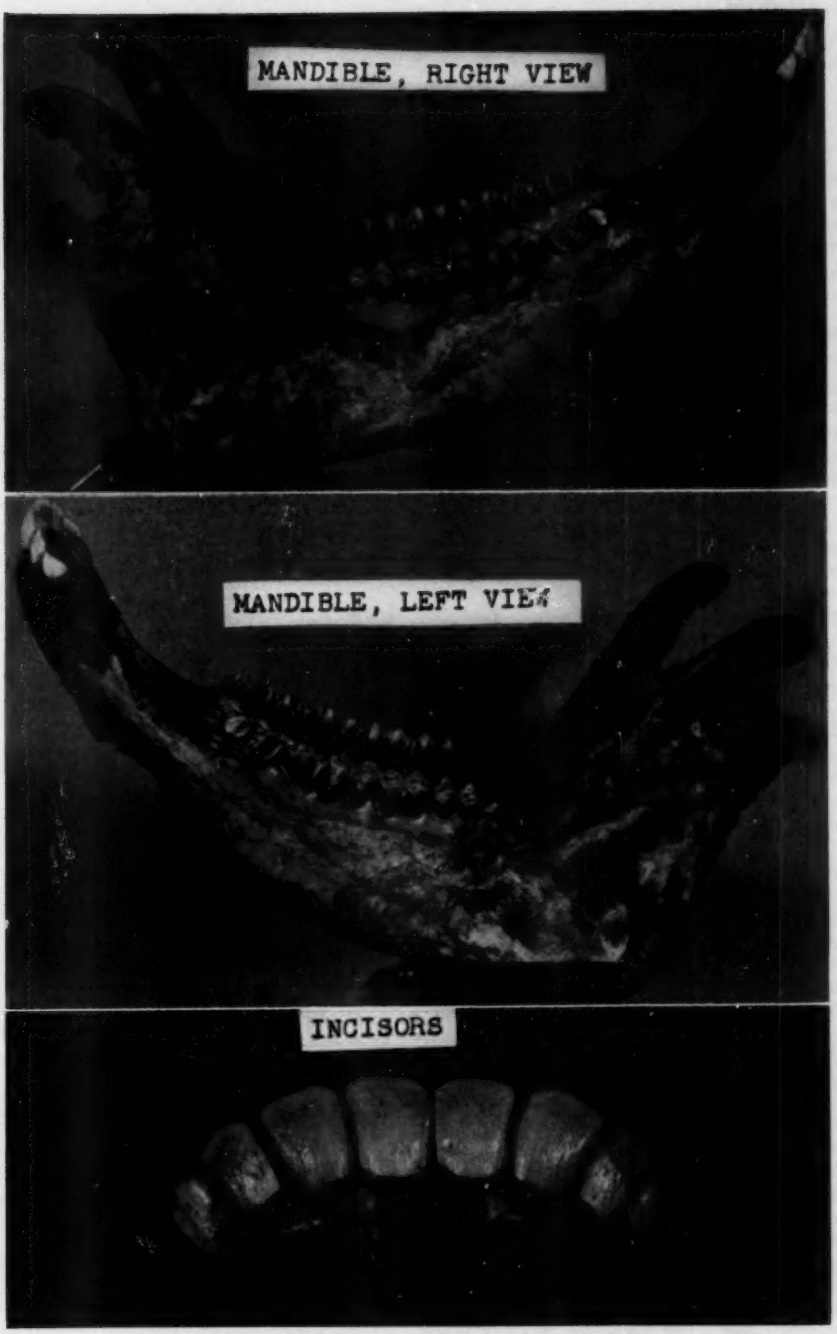


Fig. 2—Teeth of heifer 122, Milky Way Hereford Ranch, Phoenix, Ariz.

carpus was 2,600 p.p.m. Postmortem examination of the carcass indicated that all of her tissues were normal. Photographs of the incisors and lower mandible are shown in fig. 2.

In view of the fact that the metacarpus contained 2,600 p.p.m. of fluorine, when no clinical signs of fluorosis were manifested, it appears that 3,000 p.p.m. of fluorine, which has been suggested by the literature as the lowest figure at which any fluoride effect can be noted, is reasonable.

THE HOMRIGHAUSEN DAIRY FARM

The Homrighausen Farm is located within a mile of the Milky Way Hereford Ranch. The herd is composed entirely of registered Holstein-Friesians, and the owner and operator of the farm has never had any reason to suspect fluoride damage to any of her cattle.

About one-third of the milking animals were selected at random and examined. Field notes for these examinations are given in table 2, together with fluorine analyses of urine where taken and a record of the fat production supplied by the owner. Representative teeth photographs may be seen in fig. 3.

Very slight or slight stain that might be attributed to fluoride was detected on one pair of incisors in each of 4 animals. There were no signs of abnormal tooth wear in any of the animals examined and no noticeable bone involvement or other indication of adverse effects due to fluoride. The general health of the animals was good and the milk production well above average. The conclusion is warranted that the amounts of fluoride absorbed by these cattle over a period as long as ten years have had no effect, except for possible occasional slight staining of the incisors.

There is only one well on the Homrighausen Farm, which is used both for watering the cattle and for irrigation. Samples of water taken in November and December, 1949, analyzed 4.8 and 5.1 p.p.m. of fluorine, respectively. A sample of forage contained 15 p.p.m., while hay grown on the place analyzed 6 p.p.m. The corn silage contained 3 p.p.m.

Calculations have been made of the daily fluoride ingestion per animal, assuming that a 1,000-lb. dairy cow will eat 35 lb. of feed and drink an average of 15 gal. of water per day. The data used in calculating the fluoride intake was as follows:

Water ....125 lb. per day at 5 p.p.m of fluorine
Forage .... 15 lb. per day at 15 p.p.m. of fluorine
Hay.... 10 lb. per day at 6 p.p.m. of fluorine
Ensilage .... 5 lb. per day at 3 p.p.m. of fluorine
Dairy mix .... 5 lb. per day at 5 p.p.m. of fluorine.

Based on the assumptions and formula previously given, it is estimated that a 1,000-lb. (453 kg.) cow will ingest 430 mg. of fluorine per day, equivalent to 0.95 mg. of fluorine per kilogram of body weight per day. This value, as in the case of the amounts derived from the Milky Way Hereford Ranch observations, is in extraordinarily good agreement with the value derived from the literature as being borderline for cattle, namely 1 mg. of fluorine per kilogram of body weight per day. The fact that some of these animals had been exposed to such concentrations of fluorine for as long as ten years serves further to strengthen the validity of the conclusions previously reached.

The average urine analysis of 9.3 p.p.m. of fluorine is in good agreement with the value of 10 p.p.m. given in the literature as borderline between nondamaging and damaging.

THE SUNCREST HEREFORD RANCH

The Suncrest Hereford Ranch, owned by Dr. Lee Scott, enjoys a reputation in Hereford breeding circles equal to that of the Milky Way Hereford Ranch. Although it was not possible to make any observations of current problems which might be related to high fluorine content waters on the Suncrest Hereford Ranch, the experiences of Drs. Scott, McCone, and A. M. Smith, in combating past fluorosis on this ranch, are worthy of note.

This operation started at its present location on Indian School Road in 1940. At that time, the sole source of water both for the cattle and for irrigation was a well, the water of which contained 18 p.p.m. of fluorine. During the ensuing four years after 1940, a malady of slowly increasing severity was observed. The first evidences were lameness and stiffness, followed eventually by a generally unthrifty condition, with eventual mild emaciation and anemia In 1945, a thorough examination of the cattle revealed that the incisor teeth of some

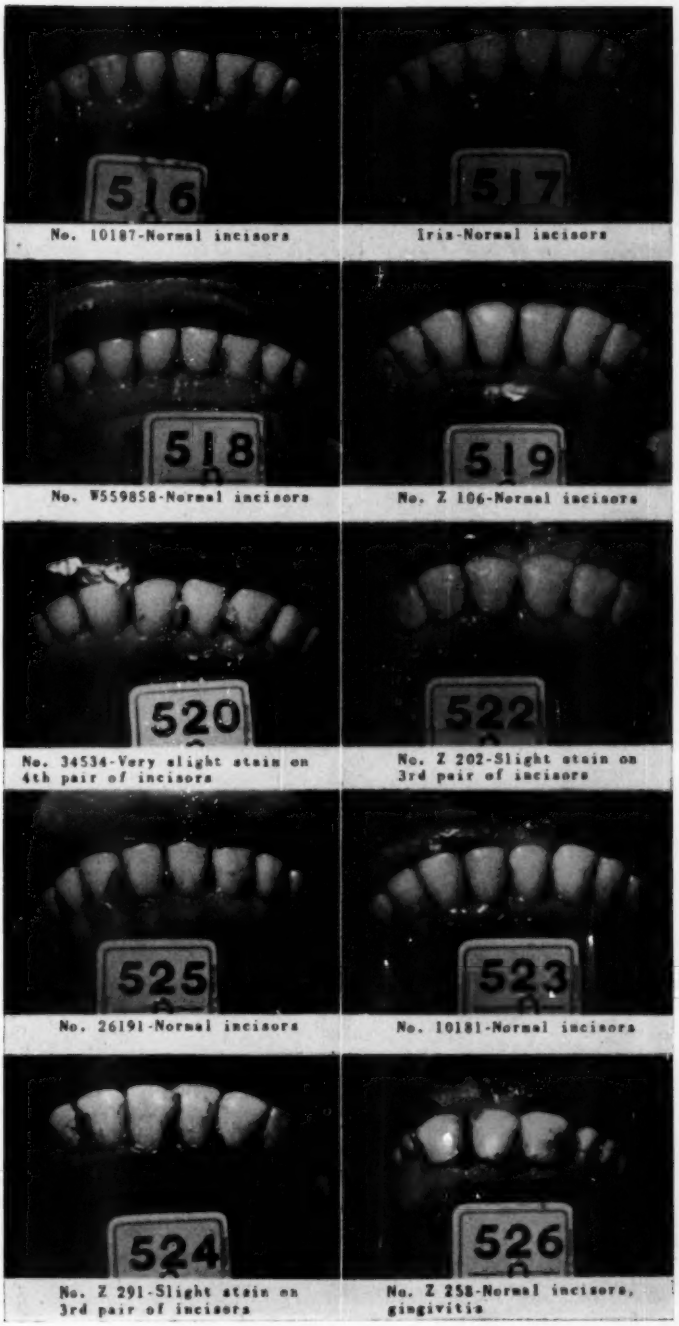


Fig. 3—Teeth of Homrighausen Dairy Farm herd, Phoenix, Ariz.

were mottled and severely worn. The malady was accordingly diagnosed as fluorosis and the water supply immediately changed, first to Roosevelt Dam irrigation water and later to Phoenix city water, both of which analyze well under 1 p.p.m. of fluorine.

When the water supply was changed in 1945 to one containing a low amount of fluorine, the trouble with the cattle started receding almost immediately. Although most of the affected animals were culled from the herd, several were retained because of their excellent breeding and nursing characteristics. Two of these that were slaughtered in 1947 and 1948 showed erosion of the surfaces of the metacarpal and metatarsal bones, with swelling and calcification of the stifle joints.

Two others of the affected animals that had been retained were slaughtered in March, 1950, and Dr. Scott made them available for postmortem examination and chemical analysis. One of these cows was somewhat anemic but bred regularly, providing ample milk for her calves, including 2 show animals. These cows showed damage to the teeth, as seen in fig. 4 and 5. The stifle joints were calcified.

Samples of urine for fluorine analysis were taken just prior to slaughter, and the metacarpal bones analyzed for fluorine. The results were as follows:

	Fluorine in urine at sp. gr. 1.040	Fluorine in metacarpus, on dry, fat-free basis
Cow 32 (11 years old, on ranch from 1940)	1.7 p.p.m.	3,360 p.p.m.
Cow 74 (9 years old, born on ranch)	3.2 p.p.m.	3,320 p.p.m.

The fluorine content of the urine was normal, showing that the system had ceased flushing off the fluorine from the skeleton. The fluorine content of the bones remained high, although it was undoubtedly much higher at the time the animals were still absorbing abnormal amounts of fluorine in 1944 and 1945. This assumption is based on the work of Blakemore *et al.*,[2] who found that a cow having a fluorine content in the bones of 8,000 p.p.m. continued to pass off abnormal amounts of fluorine in the urine for eight months after being taken off a high fluorine diet. At the end of that time, the fluorine content of the ribs had dropped to 4,000 p.p.m.

Since it is not known what the fluorine content of the forage in the Suncrest pastures was at the time the pastures were irrigated with water containing 18 p.p.m. of fluorine, it is not possible to calculate the total daily ingestion of fluoride from feed and water that must have been occurring at that time. The amount from the drinking water alone, however, would be 2.1 times as much as that received from water by a Milky Way animal spending half-time on each of the Milky Way Hereford Ranch farms. Therefore, it would appear that the Suncrest animals were probably ingesting a total of about 2 mg. of fluorine per kilogram of body weight per day.

It appears, therefore, that fluorine ingestion to the extent of about 2 mg. per kilogram of body weight per day resulted in severe fluorosis, with a bone content of fluorine possibly as high as 6,000 or 7,000 p.p.m. or higher after five years. When the source of fluoride was eliminated, the animals recovered to a degree, although some stiffness and calcification of the joints remained. Five years after the high fluoride water had been eliminated, the fluorine content of the urine was normal and the fluorine content of the bones was 3,300 to 3,400 p.p.m.

THE VARIABILITY OF THE FLUORINE CONTENT OF WELL WATERS

The question naturally arises as to whether or not the wells supplying the water on the Milky Way Hereford Ranch, the Homrighausen Dairy Farm, and the Suncrest Hereford Ranch have been substantially constant with respect to fluorine content. This question was emphasized at the time of the herd examination in December, 1949, since three samples of water from the Milky Way Hereford Ranch taken during November and December ranged from 13.4 to 17.3 p.p.m. of fluorine.

According to the foreman of the Milky Way Hereford Ranch, a sample of water from their watering well on the Home Farm was analyzed in 1945 or 1946 and was reported to contain 18 p.p.m. of fluorine. This would indicate that there was either a slight discrepancy in the results that two different analytical laboratories might find or that there had been a slight drop in

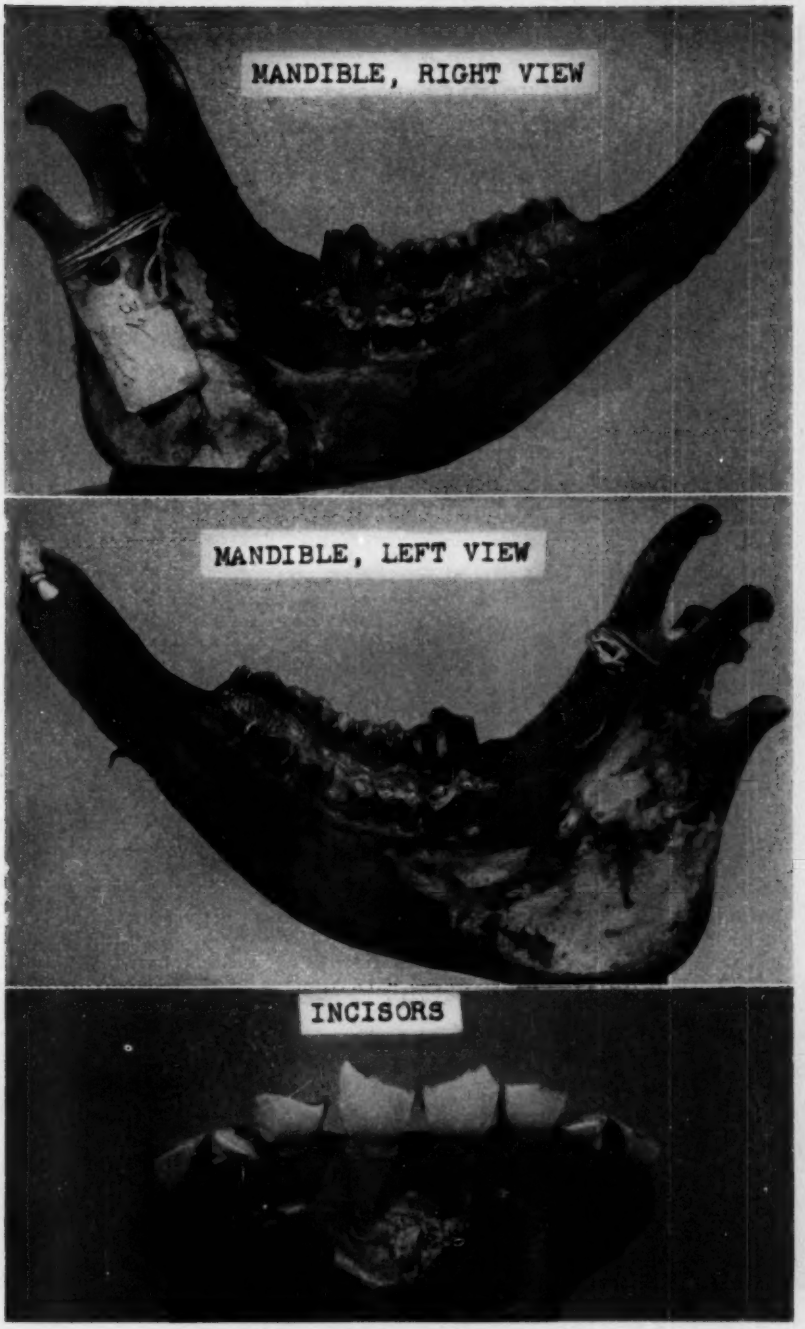


Fig. 4—Teeth of cow 32, Suncrest Hereford Ranch, Phoenix, Ariz. Portion of fourth cheek tooth scraped.

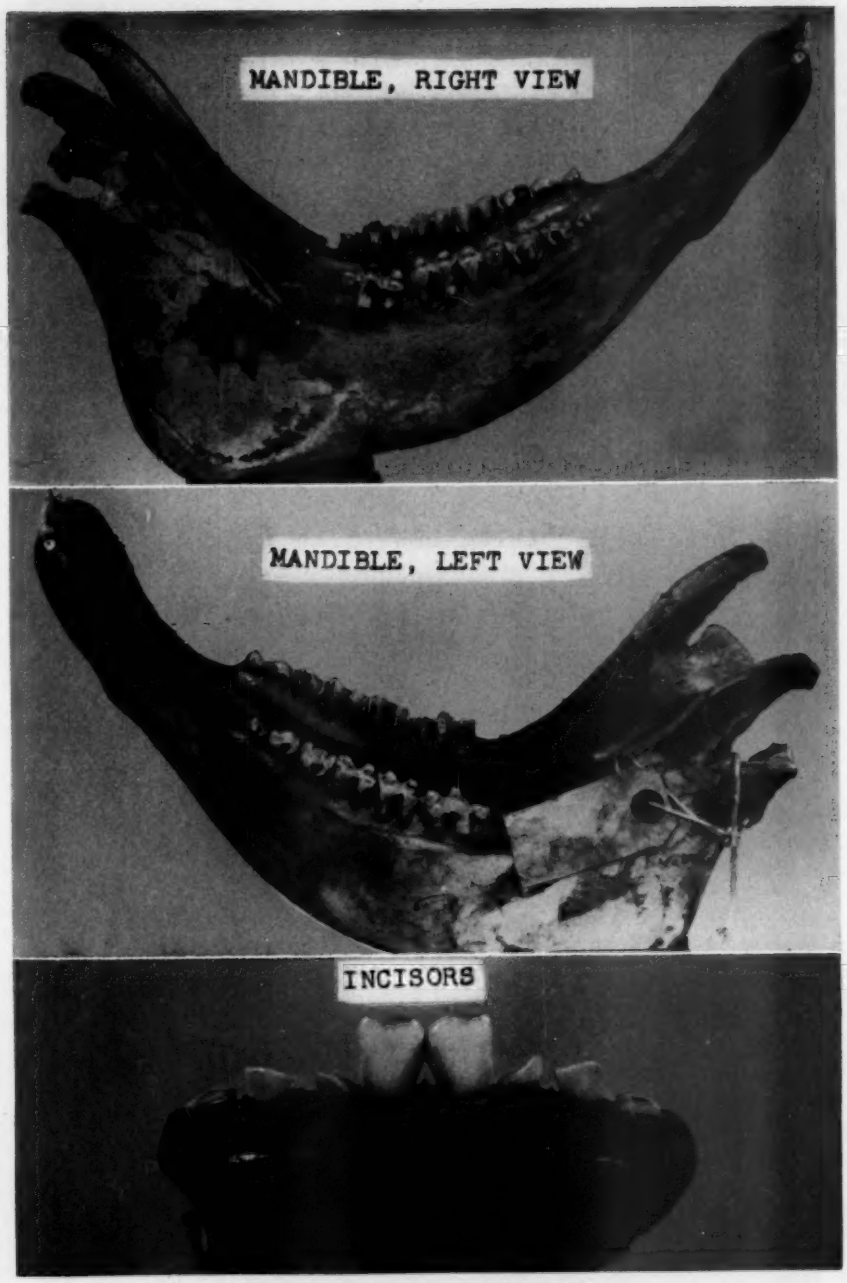


Fig. 5—Teeth of cow 74, Suncrest Hereford Ranch, Phoenix, Ariz.

fluorine content over the years. The latter hypothesis is not unreasonable, since the continued drainage of water through an aquifer would presumably slowly leach out the available fluorine-containing mineral that acted as the source of the fluoride. Also, since it is the more shallow wells in Township 2N Range 3E that exhibit high fluorine content of the water, it can be expected that, as the water drain continues, the water table will be lowered, with the result that the wells will be drawing their water from the lower aquifers where the fluorine content is lower.

Subsequent to the examinations made in December, 1949, the authors have obtained a number of additional samples of water from the Home Farm of the Milky Way Hereford Ranch, the analyses of which indicate that the fluorine content is fairly constant in the ten months covered. The various analyses on the Home Farm well are as follows:

November, 1949	17.3 p.p.m. of fluorine
December	13.4 p.p.m. of fluorine
December	15.8 p.p.m. of fluorine
April, 1950	16.4 p.p.m. of fluorine
May	16.6 p.p.m. of fluorine
July	16.2 p.p.m. of fluorine
August	16.8 p.p.m. of fluorine
Average	16.1 p.p.m. of fluorine

SUMMARY

Observations of cattle that have been continually ingesting and absorbing fluoride from drinking water and from forage provide a direct confirmation of conclusions that have been derived from a study of the literature.

Levels of soluble fluoride in the diet can be tolerated as high as 1 mg. per kilogram of body weight over periods as long as from five to ten years, with no detectable effect except possible slight mottling and slight wear of the incisor teeth.

A level of soluble fluoride in the diet of the order of 2 mg. per kilogram of body weight per day may result in mottling and severe wear of incisor teeth that form during the period of ingestion, as well as lameness and stiffness, emaciation, and general unthriftiness.

Conditions as observed in the Phoenix, Ariz., area substantiate conclusions previously drawn from the literature with respect to fluorine content of urine and bones as criteria of damage. It is concluded that the fluorine content of urine must be well above 10 parts per million to indicate current consumption of fluorine in damaging amounts. It is also concluded that the fluorine content of the metacarpus must be above 4,000 parts per million to indicate an accumulated amount that would have any effect other than some slight mottling or wear of the incisor teeth.

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The Influence of Aging on the Potency of Mammalian Intradermal Tuberculins

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THE POSSIBILITY that the potency of intradermal tuberculins declines as they age has long been a matter of concern to workers in the field of tuberculosis. An excellent review of most of the earlier work on this subject has been given by Gottschall and Bunney[1] in the introduction to a study of diluents for stabilizing tuberculins. Many of the earlier workers detected losses in potency while others did not. However, a wide variety of conditions of storage and of methods of testing for changes in potency was used. McGirr[2] reported on a study of certain added substances (e.g., antioxidants) as stabilizers of the potencies of various purified precipitated derivative (PPD) tuberculins. Long, Aronson, and Seibert[3] studied the variability of standard tuberculins in general in connection with the use of PPD. It has long been considered that the concentrated product known as Koch's old tuberculin (OT) remains potent indefinitely.[4]

Work on the stability of Bureau of Animal Industry (BAI) mammalian intradermal tuberculin has been carried on in the BAI during the last few years, because some workers have believed it desirable to increase the expiration date of the BAI tuberculins. The date has been set at one year from the date of preparation. This paper gives data which indicate that the potency decreases about 15 per cent per year; that is, the potency will decrease to 85 per cent the first year, to about 72 per cent the second year, and so on to about 50 per cent the fourth year. According to a report by Dorset[5] on field work with different dilutions of the same synthetic medium tuberculin, a decrease in potency, such as would occur in the first year, would not affect field results.

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EXPERIMENTAL

Lots of BAI mammalian intradermal tuberculins of varying ages were used in four experiments carried out from the summer of 1944 to the spring of 1950. The tuberculins were produced by the method described by Dorset and Henley[6] from cultures of human tubercle bacilli grown on the synthetic medium first described by Henley.[7] Each lot of tuberculin was derived from approximately 2,400 cultures, each grown on 100 ml. of medium. Except for such standardization as resulted from a uniform procedure which has been strictly adhered to for eighteen years, the potencies of the tuberculins were not standardized at the time of their preparation. However, skin tests were made with each lot of tuberculin on guinea pigs, for the purpose of assuring the workers in the Bureau that the tuberculins possessed potency but not for the purpose of standardizing the various lots. The samples studied in these experiments had been stored under either laboratory (experiments 1, 2, and 3) or field conditions (experiment 4) in vials sealed with a special sealing compound. Each vial was of approximately 15-ml. capacity, and each held about 12 ml. of tuberculin.

At least three concentrations of each of the tuberculins were tested by the procedures described by Johnson for testing the potency of johnins[8] and as applied in designs by Wadley.[9, 10] Amounts of 0.2 ml. were injected intradermally into cattle artificially sensitized with heat-killed, acid-fast organisms injected intraperitoneally. Measurements of skin thickness at selected sites were made prior to injection and again after forty-eight hours with the dermal thickness gauge of Kummer and Johnson.[11] The increase in skin thickness resulting from the injections was used for evaluating the potencies of the different tuberculins by means of the biological assay method as adapted to acid-fast allergen investigations by Wadley.[9, 10].

Tuberculins of recently prepared lots were used as standards. The potencies of the older tuberculins were compared with the potency

of the standard assumed as unity. For these comparisons, potency determinations were based on the fact that the relation between the logarithms of the concentrations and the responses obtained from the injections of several graded dosages is linear at moderate levels of responses. The particular concentration of a tuberculin required to produce a given response could be estimated and compared with that required for the standard. The difference between the logarithm of the concentration of the unknown required to give a selected response and the similar logarithm of the standard was the logarithm of the ratio of the potency of the unknown to the potency of the standard. By graphic means, the values thus obtained for all the different unknowns could be used to estimate the rate at which the potency decreased with age. If the logs of the potency ratios were plotted against the ages of the various tuberculins, the rate at which these logs decreased could be obtained. Subtracting the rate at which the logs decreased from 0.0 (log of unity) gave the log of the fraction of the initial potency remaining at the end of any given year. From this value, the percentage of loss in potency per year could be found (values given in the last lines of tables 1 to 4). An example which may help to make clear the rate of loss follows: a 12 per cent rate of loss is indicated; 100 per cent—(12% of 100) = 88 per cent of the original potency is present at the end of the first year; 88 per cent—(12% of 88) =77.5 per cent of the original potency is present at the end of the second year; and 77.5 per cent—(12% of 77.5) = 68 per cent of the original potency is present at the end of the third year, etc.

Further details concerning the tuberculins used, the methods of testing, and the animals used are given below in the description of each of the four experiments.

Experiment 1 (*1944-45*).—Two parallel series of tuberculins, series A and B (*see* tables 1 and 2), were tested at 1 : 40, 1 : 400, and 1 : 4,000 OT. These tuberculins had been stored in the laboratory for various periods after their preparation. Fifty cattle were divided into five equal groups. Each group was sensitized by means of an intraperitoneal injection of a mineral oil suspension of 3 Gm. (moist weight) of one of the following heat-killed, acid-fast organisms: *Mycobacterium tuberculosis* var. *bovis*, *Mycobacterium tuberculosis* var. *hominis*, *Mycobacterium tuberculosis* var. *avium*, *Mycobacterium paratuberculosis*, or *Mycobacterium phlei*. Five animals of each sensitization were used for each series of tests, or 25 animals per series. Each concentration of each tuberculin was tested on the same spot of the 25 animals. The different concentrations of the same tuberculin were placed adjacent to each other. Sites of average sensitivity were used. (*See* Wadley[9] for discussions of the variations in sensitivity of the various parts of the skin of a cow.) The results are shown in tables 1 and 2. The data on the *phlei*-sensitized animals are omitted because of the very poor reactions obtained on them.

Experiment 2 (*1948*).—Instead of testing individual lots of tuberculins of different ages, aliquots of five lots of tuberculins produced during a given year were pooled for test as a single material, since the need for testing other materials limited the number of sites on each animal which could be devoted to the present study. The pools represented tuberculins whose ages were less than 1 year year and 3, 6, 9, and 12 years. Tests were made of each of the pools at concentrations of 1 : 40, 1 : 160, 1 : 640, and 1 : 2,560 OT on the 10 cattle artificially sensitized to *M. tuberculosis* var. *bovis*. Tests were also made of each of the pools at concentrations of 1 : 10, 1 : 40, 1 : 160, and 1 : 640 OT on 10 cattle artificially sensitized to *M. paratuberculosis*.

The injections were placed at random, rather than on the same spot each time as in experiment 1. The animals were sensitized by the method already mentioned. Results are shown in table 3.

Experiment 3 (*1949*).—Aliquots of every lot of tuberculin produced in a selected year (usually 13 lots, but in one case 18 lots) were pooled for test as a single material in this experiment. The pools represented lots whose ages were less than 1 year and 1 to 2, 2 to 3, 5 to 6, and 9 to 10 years. Ten *bovis*-sensitized and 10 *paratuberculosis*-sensitized cattle were used. Concentrations of each pool of

TABLE 1—Experimental I, Series A (1944-45). Effect of Aging of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Lot (No.)	Age (years)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle	*Hominis*-sensitized cattle	*Avian*-sensitized cattle
820 Standard	<1	1.00	1.00	1.00	1.00
810	1	0.68	1.00	0.37	0.52
800	1	2.18	1.41	0.69	0.55
119R2	1	0.72	1.38	1.29	0.68
780	2	0.41	0.54	0.42	0.35
773	2	0.54	0.42	0.30	0.12
764	3	0.46	0.87	0.33	0.55
706	5	0.47	0.50	0.47	0.37
697	6	0.37	0.63	0.34	0.17
666	7	0.12	0.37	0.24	0.28
655	7	0.05	0.55	0.15	0.09
643	8	0.23	0.59	0.13	0.34
600	9	0.20	0.31	0.26	0.12
495	9	0.13	0.05	0.11	0.13
457	10	0.23	0.45	0.31	0.08
296	11	0.08	0.10	0.04	0.02
Loss of potency per year (%)		20	16	12	21

*Expressed as the ratios of the potencies of the older tuberculins to the potency of the standard, which was less than one year old. The tuberculins were stored in the BAI Washington, D. C., laboratory.

1 : 16, 1 : 64, 1 : 256, and 1 : 1,024 OT were injected at random into the skin of the *bovis*-sensitized animals, while concentrations of 1 : 4, 1 : 16, 1 : 64, and 1 : 256 OT were injected into the *paratuberculosis*-sensitized animals. The results are shown in table 4.

TABLE 2—Experiment 1, Series B (1944-45). Effect of Aging of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Lot (No.)	Age (years)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle	*Hominis*-sensitized cattle	*Avian*-sensitized cattle
820 (Standard)	<1	1.00	1.00	1.00	1.00
822	1	0.79	0.51	0.79	0.63
812	1	1.05	1.17	1.14	1.26
119R2	1	1.55	0.95	1.13	0.56
793	1	0.55	0.46	0.63	1.12
782	2	0.58	0.71	0.63	0.51
773	3	0.58	0.60	0.28	0.20
710	5	0.06	0.26	0.10	0.07
698	6	0.07	0.51	0.18	0.17
657	7	0.23	0.18	0.16	0.10
641	8	0.11	0.23	0.14	0.08
618	8	0.39	1.40	0.18	0.21
601	9	0.32	0.22	0.23	0.09
500	9	0.18	0.24	0.24	0.19
490	9	0.17	0.16	0.10	0.03
306	11	0.05	0.05	0.06	0.03
Loss of potency per year (%)		19	15	20	24

*Expressed as the ratios of the potencies of the older tuberculins to the potency of the standard, which was less than one year old. The tuberculins were stored in the BAI Washington, D. C., laboratory.

Experiment 4 (1950).—To study the effect of aging on the potency of tuberculins kept under field conditions, samples of BAI tuberculins were secured through the courtesy of the Bureau's Brucellosis and Tuberculosis Eradication Division. Sixty-nine samples were submitted from field stations, where they had been stored since their arrival from the Bureau laboratories at Washington, D. C. The field stations were in widely scattered states. Aliquots of all samples of a given lot, irrespective of the state in which the samples had been stored, were pooled for each lot represented. Then, aliquots of all lots produced in a given year were pooled for each year represented. Finally, to test representative aliquots of each sample submitted on the limited number of skin sites available on each animal, aliquots of the pools representing three successive years were combined to give three-year average pools.

As a standard, aliquots of the 35 different lots produced from early in 1947 to August, 1949, and stored in the laboratory at Washington, D. C., were pooled and considered to have an age of 1⅓ years.

The pools of the field tuberculins were considered to represent tuberculins having average ages of 2, 5, 8, 11, 14, and 17 years. Each pool was tested at concentrations of 1 : 40, 1 : 60, 1 : 90, and 1 : 135 OT on each of 10 *bovis*-sensitized cattle and at concentrations of 1 : 10, 1 : 15, 1 : 22.5, and 1 : 33.75 OT on each of 10 *paratuberculosis*-sensitized cattle. Placements were selected at random. The results are shown in table 5. Later, the identical pools were tested on each of 6 of the same group of *bovis*-sensitized animals and on each of 5 of the same group of *paratuberculosis*-sensitized cattle. In this test, however, an attempt was made to avoid extremes in reactions produced. Therefore, it was necessary to use lower concentrations on the highly sensitized animals and higher concentrations on the poorly sensitized animals than were used in the first part of this experiment. Results of this part of the experiment are shown in table 6.

Discussion of Results

Experiment 1 (tables 1 and 2) showed that the older tuberculins were less potent than the newer ones. Irregularities appeared in the downward trend of the relationship between the potencies and the ages of the tuberculins. Such irregularities were expected because of possible inequalities of potencies of the different lots of tuberculin at the time of their preparation. As mentioned before, the different lots had been standardized only by strict adherence to a uniform procedure for their preparation. Differences in potencies of the younger lots emphasized the probability that some irregularities were to be expected in the general downward trend on aging. However, the relative potencies of the older tuberculins were not the same on the different groups of animals. This fact was disturbing with respect to certain tuberculins, but these cases were not numerous enough to prevent the conclusion that the downward trend was real. The values given for the estimated rate of decrease in potency on

TABLE 3—Experiment 2 (1948). Effect of Aging on the Potency of Pools of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Age of pool (years) (5 lots of tuberculin per pool)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle
1 (Standard)	1.00	1.00
3	1.32	0.90
6	0.50	0.47
9	0.47	0.38
12	0.47	0.22
Loss potency per year (%)	11	12

*Expressed as the ratios of the potencies of the older pools to the potency of the youngest. The pools were stored in the BAI Washington, D. C., laboratory.

aging for the tests on the different sensitizations did not agree. It was believed that the method of testing was not accurate enough to assure closer agreement than was obtained.

TABLE 4—Experiment 3 (1949). Effect of Aging on the Potency of Pools of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Age of pool (years) (13 to 18 lots of tuberculin per pool)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle
1 (Standard)	1.00	1.00
1- 2	0.81	0.45
2- 3	0.50	0.26
5- 6	0.72	0.39
9-10	0.29	0.32
Loss potency per year (%)	11	11

*Expressed as the ratios of the potencies of the older pools of tuberculins to the potency of the standard pool. The pools were stored in the BAI Washington, D. C., laboratory.

Experiment 2 (table 3) was carried out with the view of smoothing out the irregularities in the downward trend of potencies with aging which, as already indicated, were possibly due to the testing of the individual lots. Pools of a number of tuberculin lots were tested in order to do this and to save testing space on the animals. The trend toward decrease in potency on aging was evident. On the *bovis*-sensitized cattle, the pools of nine- and twelve-year-old tuberculins were not as low in potency as expected. Percentages of decrease of potency from that at the beginning of any selected year were not as great as those for most of the sensitizations used in experiment 1.

Experiment 3 was a further attempt to overcome the fluctuations which were encountered when individual lots of tuberculin were tested. More lots were included in each pool than in experiment 2, but it was evident that irregularities in the downward trend were not avoided by pooling. The great difference in the ratios of the potencies of the pools on the two groups of cattle made it evident that the method was lacking in the precision required for determining a satisfactory estimate of the rate of decreases of potency on aging when only a few materials were being tested.

The data of experiment 4 were perhaps more desirable for use in determining esti-

TABLE 5—Experiment 4A (1950). Effect of Aging on the Potency of Pools of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Age of pool (years)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle
1⅛ (Standard)**	1.00	1.00
2	0.58	0.81
5	0.29	0.56
8	0.37	0.49
11	0.20	0.48
14	0.10	0.25
17	0.06	0.22
Loss potency per year (%)	15	8

*Expressed as the ratios of the potencies of the older pools to the potency of the youngest pool. The pools were stored at BAI field stations.
**Standard pool stored in BAI Washington, D. C., laboratory.

TABLE 6—Experiment 4B (1950). Effect of Aging on the Potency of Pools of Bureau of Animal Industry Mammalian Intradermal Tuberculins*

Age of pool (years)	*Bovis*-sensitized cattle	*Paratuberculosis*-sensitized cattle
1⅛ (Standard)**	1.00	1.00
2	0.60	0.20
5	0.60	0.40
8	0.30	0.80
11	0.30	0.30
14	0.20	0.20
17	0.05	0.03
Loss of potency (%)	16	19†

*Expressed as the ratios of the potencies of the older pools to the potency of the youngest pool. The pools were stored at BAI field stations.
**Standard pool stored in BAI Washington, D. C., laboratory.
†Value questionable; ratios out of line.

mates of the rate of decrease of potencies on aging than those of either experiment 2 or 3. However, in experiment 4B, on *paratuberculosis*-sensitized cattle, the values given for the ratios of potencies of the two- and eight-year-old pooled tuberculins deviated so far from the general trend and differed from their analogous values on *bovis*-sensitized cattle and from their analogous values of experiment 4A that the estimate for the rate of decrease of potencies on aging (19%) was considered suspect.

The results on the *bovis*-sensitized cattle were of more interest than those of any of the other groups because the BAI tuberculins were used in the campaign to eradicate tuberculosis from the cattle population of the United States. The average for the six estimates of the rate of decrease of potency on this group was 15 per cent. (The average for all groups including the estimate considered suspect was 16%.)

Acceptance of this estimate of loss of potency on aging means that, at the end of

the first year after preparation, the potency of a tuberculin is about 85 per cent of the initial potency; at the end of the second year, the potency is about 72 per cent of the original; at the end of the third year, it is about 61 per cent of the original; until, at the end of the tenth year, the remaining potency is only about 20 per cent of the original.

The factors involved in the loss of potency are unknown. The active principles of tuberculin are protein in nature, and aging may be considered as a physical treatment of these principles, perhaps altering them in some way so as to lower their potency. It is even possible that the aging process may cause the fractions of the tuberculin which are specific for *tuberculosis*-sensitized animals to lose potency at a rate which differs from that at which the nonspecific fractions lose potency. However, the results reported here do not indicate a consistent difference in the rate of loss of potency of the two types of fractions.

From the data obtained in these experiments, it seems probable that tuberculins should not be assumed to remain completely stable in the concentrated form. Although even the oldest tuberculins used here are capable of eliciting strong reactions in most hypersensitive cattle when they are injected at full intradermal strength (1 : 4 OT), they would undoubtedly elicit reactions in fewer tuberculous cattle than would tuberculins less than a year old. The risk of missing tuberculous cattle by using aged tuberculins should not be taken when younger products are available.

SUMMARY

The potencies of the Bureau of Animal Industry (BAI) mammalian intradermal tuberculins (or pools of those tuberculins) of various ages, up to seventeen years, have been compared with the potencies of BAI tuberculins about one year old, as standards on cattle artificially sensitized to various types of heat-killed, acid-fast microörganisms. Data are given which indicate a decrease of potency on aging of the tuberculins when they are stored at intradermal strength (1 : 4 OT) under laboratory or field conditions.

The rate of this decrease is estimated to be about 15 per cent per year from the data obtained on the groups of cows sensitized to *Mycobacterium tuberculosis* var. *bovis*. Thus, at the end of its first year of storage, it would appear that the potency of a tuberculin is probably only 85 per cent of that at the time of its preparation; further, the potency is probably about 72 per cent of the original at the end of the second year of storage, 60 per cent of the original at the end of the third year, and so on down to about 20 per cent of the original potency at the end of the tenth year.

In view of the evidence presented here, it would seem desirable that only young preparations of tuberculins be used in the campaign to eradicate tuberculosis from the cattle population, because of the possibility of missing diseased animals if the weaker, older preparations are used. The present expiration dating of one year seems justified.

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Studies in Swine Brucellosis. I. The Pathogenesis of Artificial Brucella Melitensis Infection

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SWINE BRUCELLOSIS is recognized as an insidious disease which takes a steady toll from the infected swine herd. In the corn belt states, infected swine are also the most common source of brucellosis in man. *Brucella suis* was accepted as the only cause of swine brucellosis until a study of the strains of Brucella isolated from human cases in Iowa[1] demonstrated that, while 63 per cent of the total strains isolated over a period of years were *Br. suis*, 13 per cent were *Brucella melitensis*. This became especially significant when it was found[2] that most of the cases in which *Br. melitensis* was isolated had no contact with goats or sheep, but did, for the most part, have direct contact with swine. In 1946, *Br. melitensis* was isolated by McNutt[3] from the tissues of slaughtered swine from herds in which the owners had had this type of infection. Other isolations since have further established *Br. melitensis* as a naturally occurring pathogen of swine.

These findings complicate swine brucellosis control, so it seemed desirable to study the pathogenesis of *Br. melitensis* infection in swine under controlled conditions. A project was therefore planned in which a number of known Brucella-free gilts would be studied intensively after artificial exposure to a field strain of *Br. melitensis* isolated from swine. Large exposure doses were to be given by three different routes and each type of exposure was to have uninoculated controls as a check on the natural spread of the infection.

GENERAL PLAN OF THE INVESTIGATION

Thirty-one gilts, 10 to 11 months old, which were known by previous clean tests and isolation to be free of brucellosis, were used to make up the experimental groups. At least one normal estrous cycle was observed before the animals were bred. As gilts showed signs of estrus recurring after breeding, they were rebred at least once. Estrus was determined by daily search with the boar. The gilts were divided as bred into three groups which differed only in the route of introduction of the infectious agent. Each group was maintained as a unit in a separate drylot until 110 days after breeding, when individual gilts were brought into a common farrowing house to farrow in a separate pen. Four days after farrowing, the sows and pigs were moved to large, bluegrass pasture lots until weaning. After weaning, certain pigs representing all of the litters were allowed to run in a large pasture lot together with the remaining sows in order to see whether or not the infection would be spread to these young pigs contacting the old sows. The sows were rebred immediately after weaning.

The field strain of *Br. melitensis* used was obtained through the efforts of Dr. E. D. Hubbard, then of the U. S. Bureau of Animal Industry, who was able, in the summer of 1947, to follow swine from a farm where human *Br. melitensis* infection was contracted to the packinghouse. He procured tissues for laboratory examination and, from an abscess in one of these animals, *Br. melitensis* was isolated by guinea pig inoculation. The first subculture of this isolation was lyophilized and, after four months' storage, was the source of the suspensions of *Br. melitensis* used for inoculation. Tryptose agar (Difco) slants were inoculated with the lyophilized material and, after twenty-four hours' incubation, the growth was washed off with tryptose diluent (0.1% tryptose in 0.5% NaCl solution) and was used immediately after standardization of the suspension for numbers by photometric means. Four gilts in each of the groups, at or shortly after breeding, were given a suspension containing 50 billion live *Br. melitensis*, and 4 (5 in group B) gilts were given 5 billion. Two gilts in each group were not inoculated and served as naturally exposed controls.

All the gilts in the three groups were bled at regular intervals (weekly for eight to ten weeks and biweekly until farrowing). These blood samples were tested by the standard tube-agglutination test, using doubling serial dilutions of serum starting at 1 : 25 and continuing until the maximum titer was determined. *Brucella abortus* antigen was used after it was demonstrated that the results were identical to those obtained with antigens prepared with

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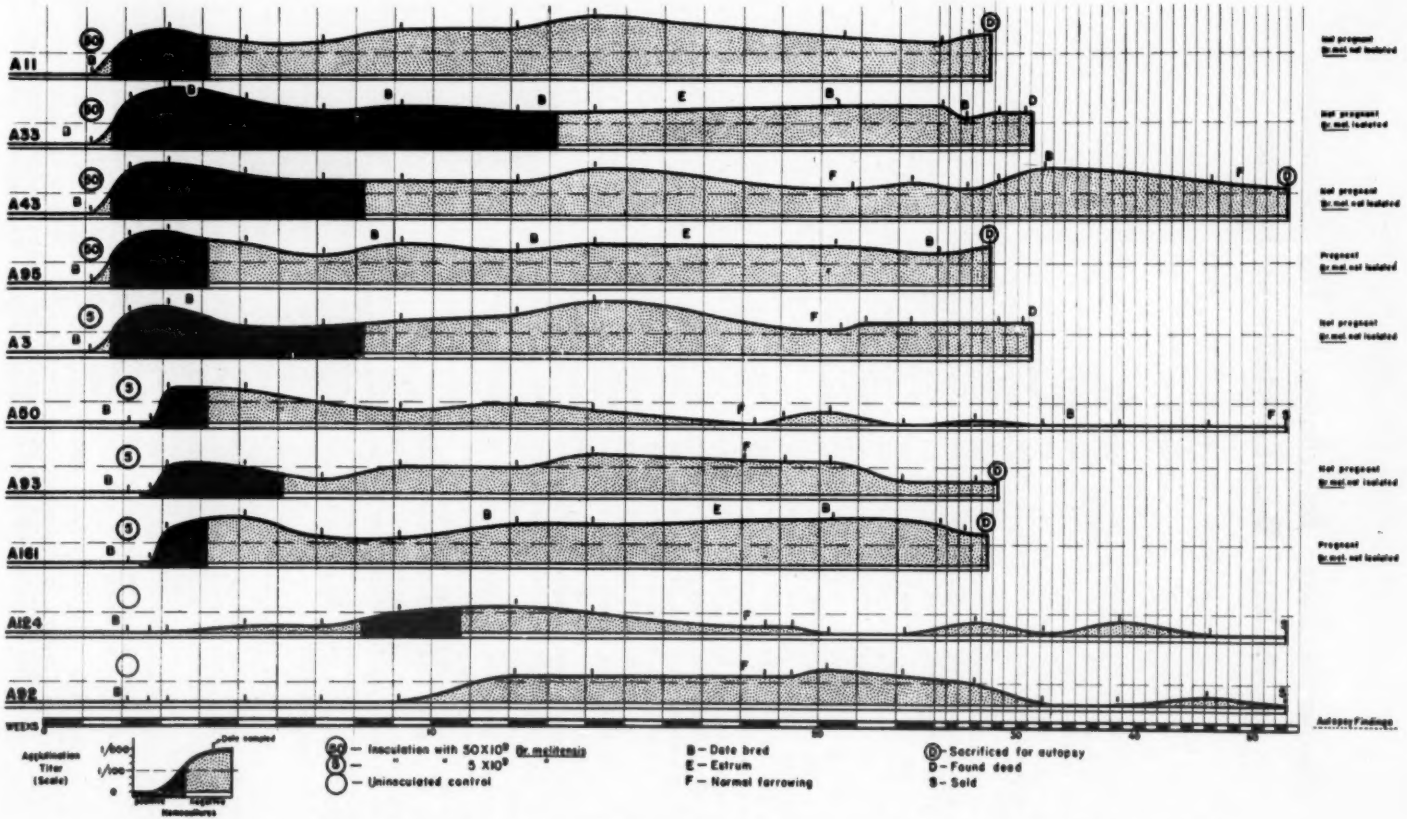

Fig. 1—Comparisons of the individual sows artificially infected with Brucella melitensis by intravenous inoculation. (Group A.)

Br. melitensis. Each blood sample was also cultured by introducing about 5 ml. of whole blood into 70 ml. of tryptose broth immediately after bleeding. These hemocultures were incubated as collected in rubber-stoppered bottles at 37 C. and subcultured on tryptose agar at weekly intervals for three weeks. Isolated cultures were identified by colonial morphology, the absence of dextrose fermentation, the inability to produce H_2S, the growth on inhibitory dye mediums, and by the ability to be agglutinated only by known Brucella-positive serums.

Blood samples from each sow were also examined three, seven, fourteen, twenty-eight, and fifty-six days after farrowing. Milk samples were collected at three and fourteen days. The flow of milk was stimulated by the intravenous injection of 1 or 2 ml. of posterior pituitary solution. After the udder of the sow was cleaned with 70 per cent alcohol, the milk was collected by hand milking from all the quarters into sterile, wide mouth, screw cap bottles. The milk was allowed to separate and after centrifuging the cream and the sediment were each cultured on tryptose agar plates. The cream was also inoculated into guinea pigs. The whey-agglutination test was made on the milk samples. Vaginal swabs were cultured on tryptose agar plates three, seven, and fourteen days after farrowing.

At farrowing, all dead pigs, 1 live pig from each litter, and the placentas were examined, cultured, and guinea pig inoculations made. Several sows were destroyed and, together with those which died during these investigations, were subjected to detailed postmortem examination to determine the status of the Brucella infection. All postmortem material was cultured on tryptose agar and inoculated into guinea pigs.

All the baby pigs were routinely bled for agglutination tests and hemocultures at 28 and 56 days of age. A few litters were also bled several times during the first days of life. After weaning, all the sows and the pigs running with them were bled at intervals to determine the course and spread of the disease.

Group A—Intravenous Inoculation.—Within a few days after breeding, 8 of the 10 gilts in this group were artificially exposed to *Br. melitensis* by intravenous inoculation of the bacterial suspension. Figure 1 is a graphic, chronological comparison of the agglutination titer curves, positive hemocultures, and the breeding history of the individual sows in this group.

All of the inoculated animals had an immediate rise in agglutination titer and all had bacteriemia lasting from two to twelve weeks after inoculation. It is noted that in most of the gilts there was a tendency for a persistent high agglutination titer. In only one of the artificially infected gilts in this group did the agglutination titer recede to negative. In those animals sampled on the third day after inoculation, bacteriemia preceded the rise in antibodies.

Gross evidence of abortion, such as fetuses or placentas, was not found, however 3 gilts did show signs of estrus eight to ten weeks after being bred and when rebred failed to conceive. Two of these gilts (A95 and A161), when rebred after a period of sexual rest, conceived and were pregnant at autopsy. One gilt (A11) never showed signs of estrus after breeding, did not farrow and, when destroyed for autopsy, showed no evidence of being or having been pregnant.

The uninoculated controls in the group both showed evidence of having contracted the infection by natural exposure. Gilt A124 had a positive hemoculture seven weeks after being added to the group and a maximum agglutination titer of 1 : 200. Gilt A92 had no demonstrable bacteriemia but did develop an agglutination titer as high as 1 : 400. Both controls farrowed normally and their agglutination titers receded to negative during the investigation.

Four of the 8 inoculated gilts farrowed normally. They had 40 pigs, of which 4 were born dead and 32 were weaned (8 pigs per litter weaned). The 2 control gilts farrowed 20 pigs, 3 of which were dead, and weaned 14 (7 pigs per litter weaned).

Twenty-six weeks after inoculation, 3 gilts (A11, A161, and A95 which had chronic lameness for several months) were destroyed for postmortem examination. No lesions were found and *Br. melitensis* was not isolated by direct culture or by guinea pig inoculation, in spite of the fact that all 3 sows had high persistent agglutination titers. Sow A93 was slaughtered a week later, since she had become extremely lame and had lost a great deal of weight. Postmortem lesions found were interpreted as a fracture of the femur with a rather severe inflammatory reaction. Pathogenic microörganisms were not isolated, and it is probable the condition was not preceded by a Brucella osteomyelitis. Thirty weeks after inoculation, sows A33 and A3 were found dead of heat prostration. *Brucella melitensis* was isolated from the cervical lymph glands, the superficial inguinal lymph glands, and from the uterine mucosa of sow A33. While sow A43 had been lame for many months and had had an agglutination titer of 1 : 200 or higher for over a year, *Br. melitensis* could not be isolated when she was slaughtered.

Group B—Conjunctival Sac Instillation.—Nine of the 11 gilts in this group were artificially exposed to *Br. melitensis* by introduction of the bacterial suspension into the conjunctival sac two to five days after being bred. The response of the individual animals to the infection is compared in fig. 2.

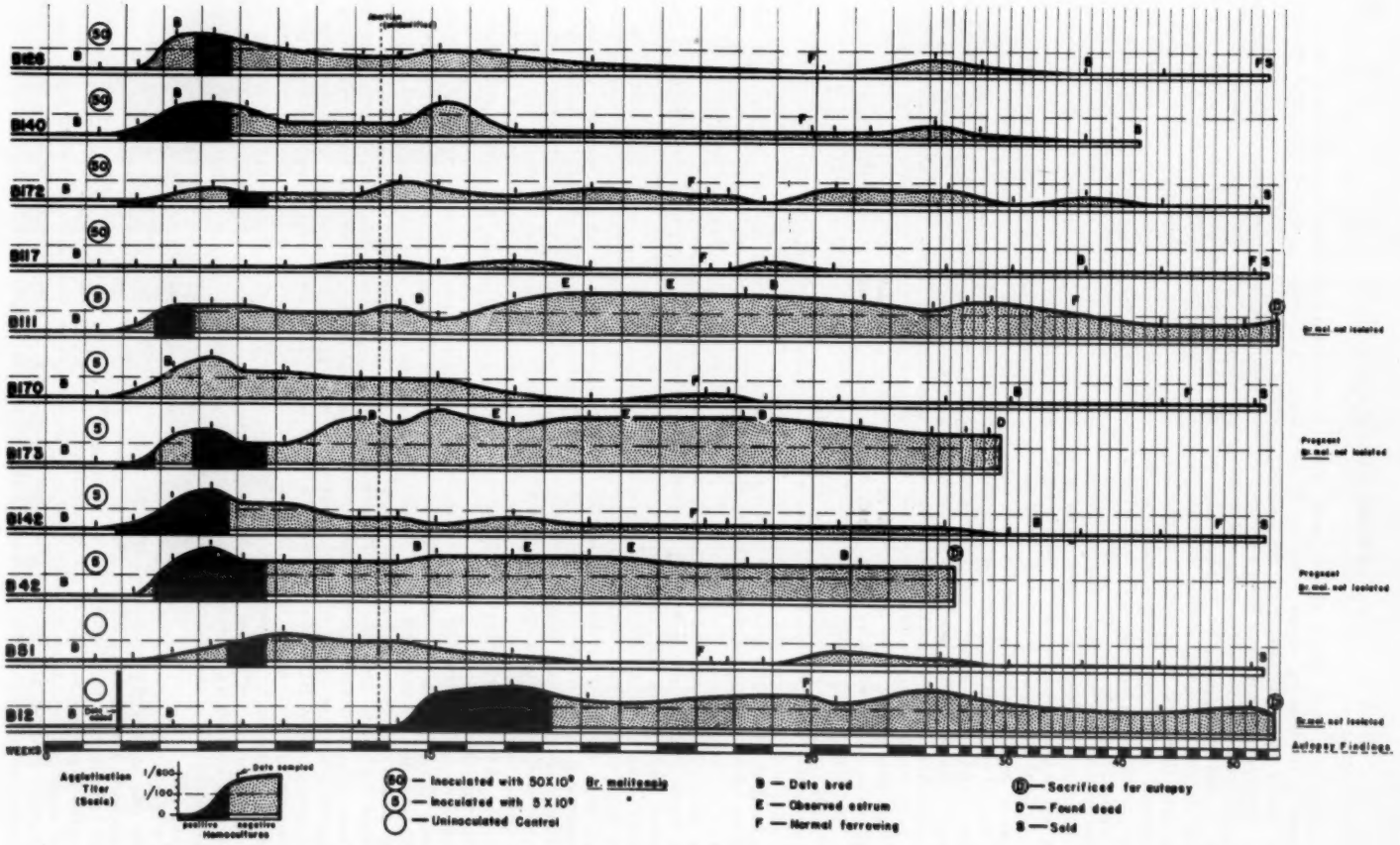

Fig. 2—Comparisons of the individual sows artificially infected with Brucella melitensis by conjunctival sac introduction. (Group B.)

With the exception of 1 animal, all the gilts had a marked agglutination response. It will be noted that, in general, the agglutination titers were lower and had more tendency to recede than in group A, so that by farrowing time the gilts which farrowed had gone down to 1 : 25 or negative. All but one of those having an agglutination response did have a Brucella bacteriemia for one to three weeks.

Seven and a half weeks after introduction of the infectious agent, a placenta was found in the lot. The identity of the aborting gilt could not be determined, but from fig. 2, it wil be noted that it could well have been gilt B111 or B42. Three gilts apparently aborted from seven to eight weeks after breeding. When rebred, they continued to come back in heat at irregular intervals. After resting through two estrous cycles, these gilts did conceive when rebred.

Infection by natural exposure was demonstrated in the 2 control gilts. Gilt B51 was placed in the group when the other gilts were inoculated. She had a positive hemoculture four weeks later and an agglutination titer as high as 1 : 200. The other control (gilt B12) was not placed in the group until four days after the inoculation of the other gilts to minimize contamination from externally placed organisms at the time of the conjunctival sac instillation. Eight weeks later, she had a bacteriemia which was followed by an agglutination titer rise to 1 : 800. It is interesting to note that this infection is timed to correspond with the known abortion in the group.

In group B, 6 of the 9 gilts artificially infected with *Br. melitensis* farrowed normally. They had 75 pigs, of which 8 were born dead and 63 weaned (10.5 pigs per litter weaned). The 2 control gilts farrowed 19 pigs, 2 of which were born dead and 14 weaned (7 pigs per litter weaned).

From twenty-six to fifty-three weeks after inoculation, 3 of the animals were destroyed and 1 died of heat prostration. No lesions suggestive of brucellosis could be found on postmortem examination. *Brucella melitensis* was not isolated from any of these animals.

Group C—Intravaginal Instillation.—Eight of the 10 gilts in this group were artifically exposed to *Br. melitensis* by introduction of the bacterial suspension into the vagina by means of a rubber catheter immediately after breeding.

It is obvious from fig. 3 that the response to the infecting agent was less than in the case of the other routes of inoculation. There was an agglutination response in all of the inoculated animals, but in some cases quite low. Three of the inoculated gilts had an immediate bacteriemia, while 1 was delayed four weeks and another eighteen weeks.

No abortions occurred although gilt C133 was not pregnant when autopsied. In contrast to the other groups where all farrowings were normal, 2 in this group were considered abnormal. Gilt C113 had difficulty farrowing and she gave so little milk that her pigs were finally allowed to rob from other sows. Her condition did not improve, and when she was killed it was found that the remains of a fetus which had broken through the uterine wall were enclosed by omentum in the peritoneal cavity. Gilt C160 also farrowed an abnormal litter: of the 10 pigs farrowed, 3 were mummified, 5 were dead, and the 2 live ones were very small and weak. *Brucella melitensis* could not, however, be isolated from either of these sows or their pigs.

The two uninoculated controls had only slight reactions to the agglutination test. They were placed in the group a week after the last artificial inoculation to lessen the chance of external contamination.

Six of the 8 inoculated gilts farrowed 66 pigs, of which 15 were dead and 46 weaned (7.7 pigs per litter weaned). The two control gilts farrowed 20 pigs, of which 2 were dead and 14 weaned (7 pigs per litter weaned).

Gilt C133 died of heat prostration following an injury. *Brucella melitensis* was isolated from the deep inguinal lymph glands. While she had showed no signs of estrus since breeding seventeen weeks before, she was not pregnant. Gilt C135 went down three weeks after breeding and, on postmortem examination, a large exostosis was found under two necrotic thoracic vertebral bodies. It could not be determined whether this was the result of an injury or an osteomyelitis caused by *Br. melitensis*, since the only organism isolated was *Corynebacterium pyogenes*.

General Observations

Much of the data collected can best be summarized in general terms, since it was not characteristic of any particular group. After artificial exposure to *Br. melitensis*, most of the gilts showed evidence of general reaction: depression, loss of appetite, and lameness. Group A had the most marked symptoms and had considerable difficulty with lameness. This lameness was shifting in nature, most severe in the coldest weather, and in some cases, animals had to be helped up for a week at a time. Groups B and C had less marked symptoms, respectively.

Brucella melitensis was not isolated from the milk samples taken routinely after farrowing. Only 5 sows (A3, A43, B117, B12, and C112) had positive whey-agglutination tests. The routine vaginal swabs taken were negative for Brucella in all cases. In

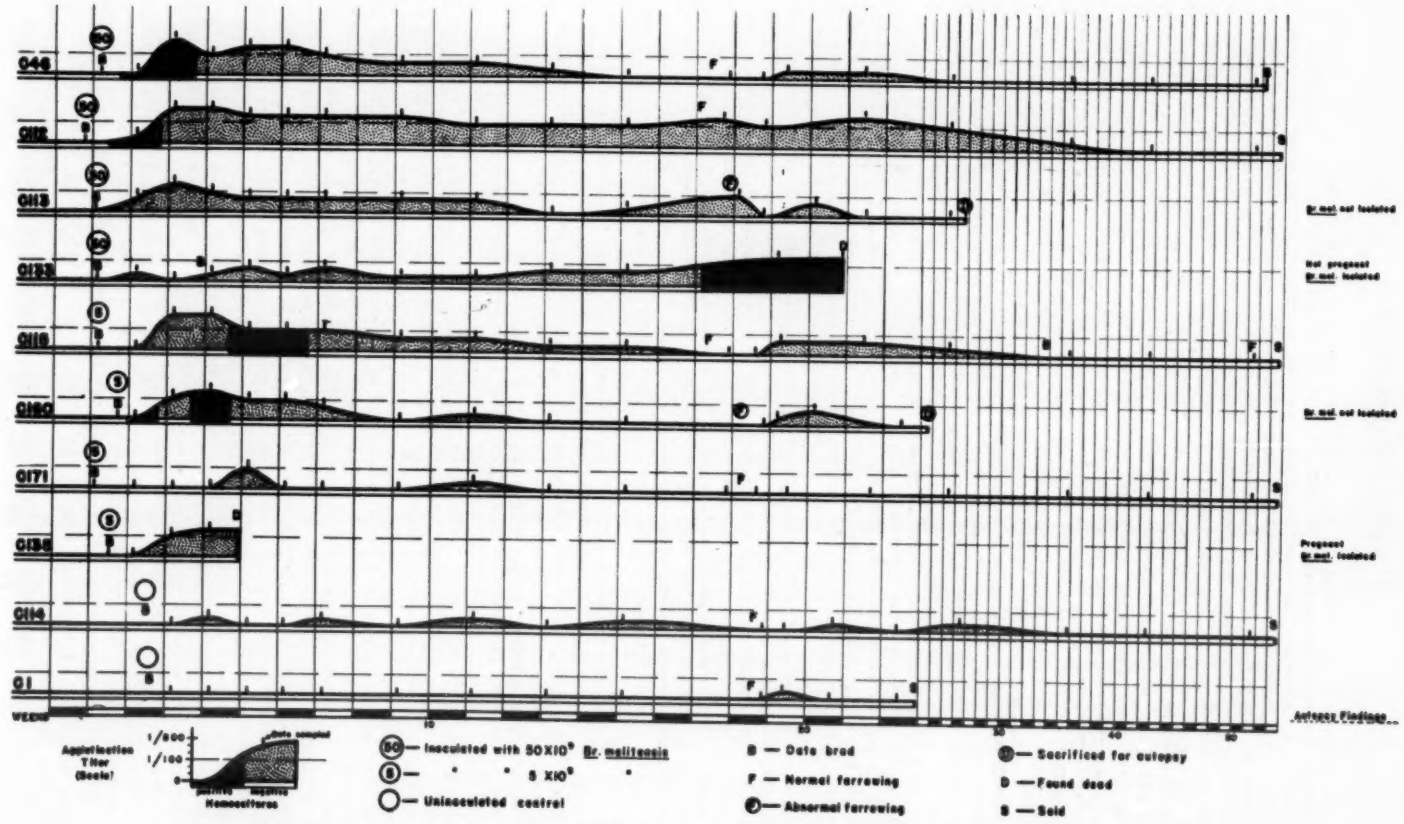

Fig. 3—Comparisons of the individual sows artificially infected with Brucella melitensis by intravaginal introduction. (Group C.)

these investigations, those gilts which did not abort farrowed normal litters of healthy pigs. *Brucella melitensis* was not isolated from any of the baby pigs examined. As expected, those examined before nursing had no agglutination titer, while the maximum titer was reached in a matter of hours after receiving colostrum. Most of the agglutination titers were dissipated by four weeks. Only 1 pig had a titer (partial 1:50) at eight weeks, and it was negative when tested two weeks later.

At weaning, 30 gilt pigs and 6 boar pigs representing all groups and litters were collected in a large pasture with the remaining sows. Many of the sows were rebred when they came into estrus. These animals were held and observed for six months. None of the pigs developed an agglutination titer during this period. Three ewes with lambs kept in this lot did not develop agglutination titers or symptoms of brucellosis. This was taken as evidence that the sows had ceased spreading the organism. It should be noted, however, that a number of sows with high agglutination titers had been destroyed or died from other causes before this phase of the investigation was in progress. The second farrowing of the old sows was entirely normal.

The four breeding boars used in this experiment did not become infected, in spite of the fact that they repeatedly bred infected gilts and, at one stage, were turned in with the "sterile" gilts for several weeks.

In studying the charts, it is noted that in a number of sows a period of instability in the agglutination titer existed after farrowing. A tendency for some agglutination titers to go up after farrowing has also been noted in some of our *Br. suis* investigations.

It would seem that the *Br. melitensis* strain used has given results similar to what would be expected from a *Br. suis* strain of moderate virulence. The exposure doses used were large and, while larger than necessary for intravenous inoculation, were barely large enough for intravaginal introduction.

SUMMARY

Thirty-one gilts were divided into three groups and exposed artificially to a field strain of *Brucella melitensis* by three different routes: intravenous, conjunctival sac, and intravaginal. Intensive studies were made to determine the pathogenesis of this type of brucellosis in swine.

Infection was accomplished by all routes of exposure used. Spread by natural means to uninoculated controls was accomplished in two of the groups.

The results of this investigation lead to the conclusion that the pathogenesis of infection of swine with *Br. melitensis* is similar to that with *Brucella suis*. The strain used in these studies seemed moderate in virulence as compared to some strains of *Br. suis*.

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On the Blood Picture of Healthy Southdown and Hampshire Ewes

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INFORMATION ON the cellular elements of sheep blood rests largely on a report from Scottish sheep. Holman[1] studied the blood picture of 171 Scottish hill sheep " . . . Cheviots and Blackfaces . . . and their crosses with the Border Leicester breed . . . Halfbreds and Greyfaces, respectively." Such information is important at this laboratory because of a series of continuing studies on the blood picture of lambs given experimental infections with medium stomach worms. Actually, the value of sheep as experimental animals in studies on host-parasite relationships is impaired because the hematology of sheep rests on such a restricted base of animals.

The present study is based on blood samples obtained from 55 purebred Southdown and 35 purebred Hampshire ewes. The data are not considered to establish "standards" but simply to add to the store of information on the hematology of sheep. The ewes themselves were the complete Southdown and Hampshire membership of the Kentucky Agricultural Experiment Station flocks from which have been produced a considerable number of prize-winning lambs at the International Livestock Exposition in Chicago. The majority of the ewes were due to lamb within a two-month period after the blood samples were taken; the blood pictures, therefore, are of pregnant ewes. None of the animals here recorded gave obvious evidence of disease and are assumed to have been normal healthy individuals.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

METHODS

All blood samples were collected between 8 and 9 a.m. Five milliliters of blood were collected from the jugular vein in tubes containing 1.3 mg. of ammonium and 0.7 mg. of potassium oxalate. Hemoglobin concentrations were measured by a Fisher electrohemometer. Wintrobe's hematocrit tubes were used for the determination of the packed volume of erythrocytes; the hematocrits were centrifuged for one hour at approximately 3,000 revolutions per minute. Counts of the red and white cells were done in duplicate using Spencer bright-line hemocytometers. The duplicate red cell counts were not recorded unless they were within 500,000 cells per cubic millimeter of one another. Duplicate white cell counts were not

TABLE I—Blood Picture of 55 Healthy Purebred Southdown Ewes

Measure	Erythrocytes (10^6/cmm.)	Hemoglobin (Gm./100ml.)	Packed volume of erythrocytes (%)	Mean cell volume (cμ)	Leukocytes (10^3/cmm.)
Range	8.27–15.51	10.3–16.0	32.3–47.2	23.6–48.6	3.15–11.88
Median	12.89	13.0	40.4	32.3	7.2
Arithmetic mean and s.e.*	12.65±0.214	13.41±0.161	40.38±0.441	32.23±0.443	7.26±0.264
Standard deviation	1.59±0.152	1.19±0.114	3.27±0.311	3.28±0.313	1.96±0.187

Leukocytes (%)

Measure	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Range	10.0–53.9	34.8–77.0	0.5–7.5	1.8–25.0	0–4.0
Median	27.0	62.5	2.3	5.5	1.0
Arithmetic mean and s.e.	27.6±1.04	62.4±1.22	2.4±0.19	6.2±0.52	1.4±0.14
Standard deviation	7.6±0.74	8.9±0.87	1.4±0.13	3.8±0.37	0.9±0.10

*Standard error

recorded unless they were within 1,000 cells per cubic millimeter of one another. The differential white cell counts were obtained from smears made with fresh blood and are based on 200 cells.

RESULTS

The results of this study are summarized in tables 1 and 2.

The average erythrocyte counts of 12,655,000 ± 0.214 million per cubic millimeter for the Southdown ewes and 12,473,000 ± 0.316 million per cubic millimeter for the Hampshire ewes are higher than the value of 11.5 million for the sheep reported by Holman.[1] The two values in our sheep also exceed the figure of 11.9±1.2 million erythrocytes per cubic millimeter of blood recently reported by Becker and Smith,[2] who studied a group of 18 sheep which was comprised of 6 lambs, 6 yearlings, and 6 mature sheep. Our average hemoglobin value for Hampshire ewes, 12.34 ± 0.195 Gm. per 100 ml. of blood, agrees with Holman's figures of 12.4 Gm., but our Southdown ewe value was 13.41 ± 0.161 Gm. per 100 ml. of blood. All three values exceed that reported by Becker and Smith, 10.2 ± 1.0 Gm. In general, each of our red cell values is somewhat higher than those recorded by Holman or by Becker and Smith. Holman's average of 9.2 thousand leukocytes per cubic millimeter of blood exceeds our average values of 7.74 ± 0.365 thousand per cubic millimeter for the Hampshire and 7.26 ± 0.264 thousand per cubic millimeter for the Southdown ewes.

Values obtained from the two breeds of sheep at this Station are in very good agreement with each other except for hemoglobin, packed cell volume, and mean cell volume. The Southdown ewes recorded had more and slightly larger red cells than the Hampshire ewes. No particular significance is attached to these differences, however, because only 55 Southdown ewes and 35 Hampshire ewes were studied.

TABLE 2—Blood Picture of 35 Healthy Purebred Hampshire Ewes

Measure	Erythrocytes (10^6/cmm.)	Hemoglobin (Gm./100ml.)	Packed volume of erythrocytes (%)	Mean cell volume (cμ)	Leukocytes (10^3/cmm.)
Range	9.52–17.80	10.4–14.7	31.3–45.2	26.7–41.9	4–13.62
Median	12.40	12.1	37.8	30.6	7.5
Arithmetic mean and s.e.*	12.47±0.316	12.34±0.195	38.35±0.661	31.07±0.505	7.74±0.365
Standard deviation	1.87±0.223	1.15±0.137	3.91±0.468	2.98±0.357	2.16±0.258

Leukocytes (%)

Measure	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Range	15.0–49.3	45.8–77.5	0.3–6.5	1.0–18.5	0.5–4.5
Median	27.5	61.5	1.8	4.3	1.8
Arithmetic mean and s.e.	28.5±1.39	62.0±1.46	2.2±0.25	5.5±0.60	1.9±0.18
Standard deviation	8.2±0.98	8.7±1.03	1.5±0.17	3.6±0.43	1.1±0.13

*Standard error.

SUMMARY

1) A study of the blood picture of 55 healthy purebred Southdown pregnant ewes revealed the following average blood values and their standard errors: erythrocytes, 12.65 ± 0.214 million per cubic millimeter; hemoglobin, 13.41 ± 0.161 Gm. per 100 ml. of blood; packed volume of erythrocytes, 40.38 ± 0.441 per cent; mean cell volume, 32.23 ± 0.443 cμ; and leukocytes, 7.26 ± 0.264 thousand per cubic millimeter.

2) The Southdown ewes differential leukocyte counts showed the following mean percentages and standard errors; neutrophils, 27.6 ± 1.04; lymphocytes, 62.4 ± 1.22; monocytes, 2.4 ± 0.19; eosinophils, 6.2 ± 0.52; and basophils, 1.4 ± 0.14.

3) A study of the blood picture of 35 healthy purebred Hampshire pregnant ewes revealed the following average blood values and their standard errors: erythrocytes, 12.47 ±0.316 million per cubic millimeter; hemoglobin, 12.34 ± 0.195 Gm. per 100 ml. of blood; packed volume of erythrocytes,

38.35 ± 0.661 per cent; mean cell volume, 31.07 ± 0.505 $c\mu$; and leukocytes, 7.74 ± 0.365 thousand per cubic millimeter.

4) The Hampshire ewes differential leukocyte counts showed the following mean percentages and standard errors: neutrophils, 28.5 ± 1.39; lymphocytes, 62.0 ± 1.46; monocytes, 2.2 ± 0.25; eosinophils, 5.5 ± 0.60; and basophils, 1.9 ± 0.18.

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The Blood Sedimentation Rate in the Horse

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WHEN WHOLE blood mixed with an anticoagulant is placed in an upright tube, the cells sink because they are heavier than the plasma in which they are suspended. As discovered by Fahraeus,[1] the suspension stability of blood is altered in pregnancy and many diseases; thus a simple test was devised which has become of considerable clinical value.

In the course of the studies of the effect of equine infectious anemia on the horse, the sedimentation rate of the red blood cells was one of the items investigated. Coffin[2] noted that the rate in the horse was extremely fast normally, and recommended reading the rate at the end of ten minutes and again at twenty minutes. He provisionally gave the rate for a normal horse, when using the Wintrobe hematocrit tube, as 2 to 12 mm. in ten minutes and 15 to 38 mm. in twenty minutes. However, it was decided to use the Westergren procedure, because the test can be run with a minimum of time and effort, the pipettes are easy to clean and fill, and the length of the tube, which is greater than in other commonly used methods, tends to minimize the error due to packing of cells.

Van Zijl[3] investigated the sedimenta-

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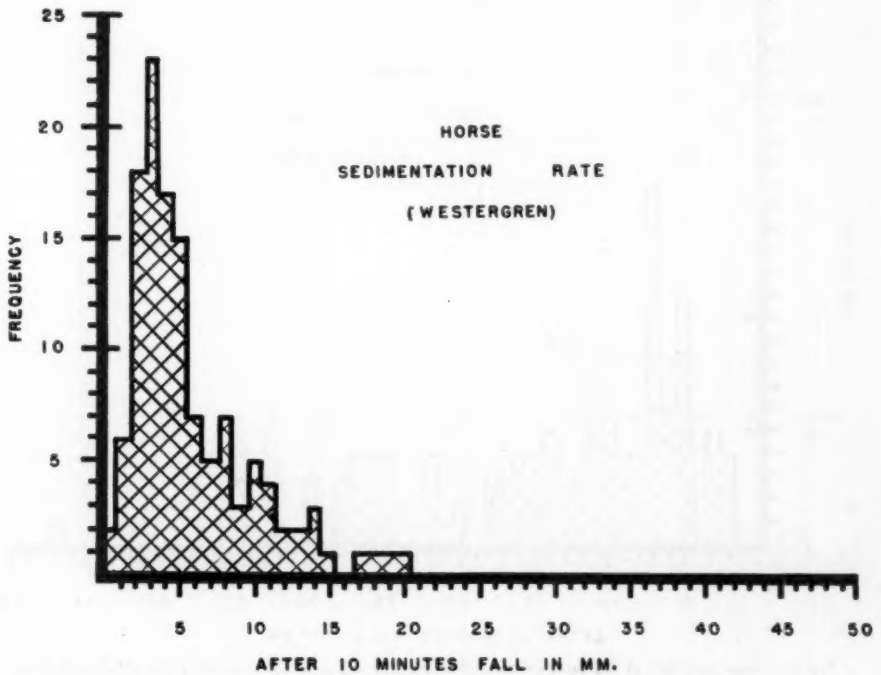

Fig. 1—For the most part, only the first phase of settling has taken place. The sedimentation rate ranges from 0 to 20 mm.; the average is 5.6 mm.

tion rate in the horse using the Westergren procedure. His results were obtained by taking two readings—the first after thirty minutes, the second after sixty minutes—and the mean sedimentation rate per ten minutes (SR/10) was calculated. By using a correction chart for red cell volume constructed for a normal of 30 per cent, the corrected SR/10 was found to lie between 21.9 ± 4.58 mm.

As a matter of convenience, it was decided to run all hematological determinations from horses infected with equine infectious anemia using a single sample of oxalated whole blood. This would mean determining the sedimentation rate on an oxalated sample instead of the usual citrated sample. A correction chart, patterned after the one prepared by Wintrobe and Landsberg[4] for human blood but based on the SR/10, was prepared. And lastly, the values were calculated on the basis of a normal red cell volume of 42 per cent, as preliminary work had shown this to be an average value for the horses under investigation.

Materials and Methods

The blood samples were collected by puncture of the jugular vein into a tube containing a dried film of ammonium and potassium oxalate, as described by Heller and Paul.[5] Five milliliters of blood were taken from which this and other hematological determinations were run. All determinations were run within one and one-half hours following collection of the blood sample.

Westergren pipettes, 300 mm. in length, 2.5 mm. bore, and graduated in millimeters up to 200, were used. The pipettes were filled to the zero mark and placed in a Westergren rack in vertical position. The rate at the end of 10, 15, 20, 30, 45, and 60 minutes was recorded. A temperature of 70 to 80 F. was maintained throughout this procedure.

For the estimation of the SR/10, the procedure outlined by Van Zijl[3] was followed. This involved two readings—the first after thirty

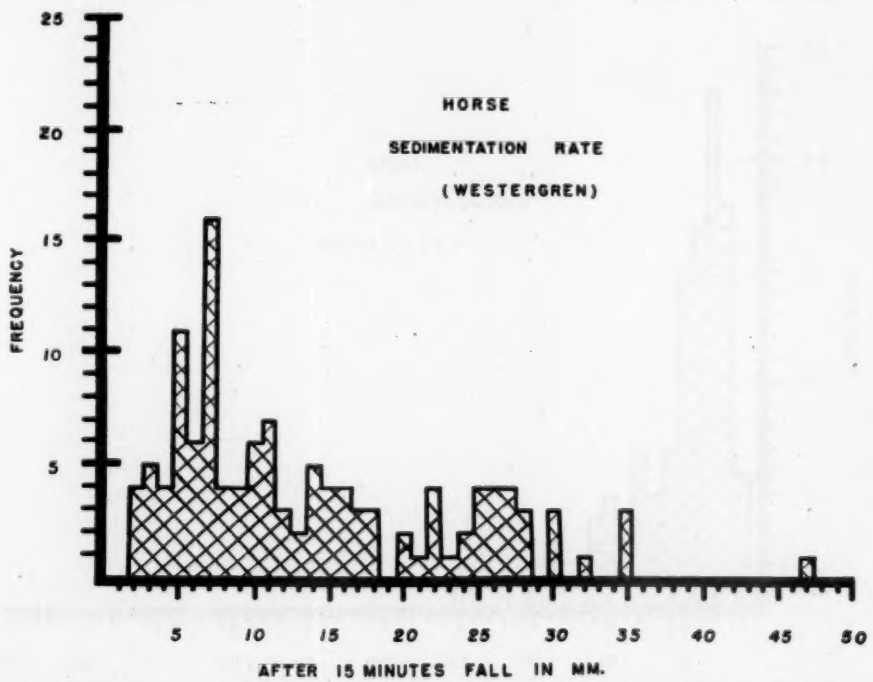

Fig. 2—About one half of the samples are still in the first phase of settling, while the other half have already entered into the phase of maximal sedimentation velocity. The average fall is 13.7 mm., while the range is from 2 to 47 mm.

minutes (a), the second after sixty minutes (b)—and the application of the modified Katz formula:

$$SR/10 = \frac{\frac{a}{3} + \frac{b}{6}}{2}$$

For the construction of a correction chart for sedimentation time, corrected for cell volume, the data obtained by Van Zijl[5] on 80 horses were utilized. (These data were prepared by rendering the samples anemic and polycythemic by diluting the blood with its own plasma or reducing its plasma quantity. In this way, samples containing 10%, 20%, 30%, 40%, and 50% of red cells were obtained. The sedimentation rate was measured and the SR/10 calculated.)

Discussion and Results

Cutler[6] has demonstrated that the process of sedimentation consists of three phases. During the first phase of settling, the cells begin to show rouleaux formation and fall slowly (phase of aggregation). This is followed by an intermediate phase of maximal fall, which becomes progressively faster until the cells begin to pack. Lastly, the phase of packing when corpuscular masses, in accumulating at the bottom of the tube, slow the rate of sedimentation.

After running 124 different determinations on 13 normal horses over a period of thirteen months, it became evident that the time required for each phase to be completed was quite variable. When these samples were compared after ten minutes of settling, it was found that the sedimentation rate ranged from 0 to 20 mm. The average was 5.6 mm. From an examination of fig. 1, it may be noted that, for the most part, only the first phase has taken place. When these same samples were examined at the end of fifteen minutes' fall, the distribution was considerably spread out. From an examination of fig. 2, it may be noted that about half of the samples are still in the first phase, while the other half have already entered

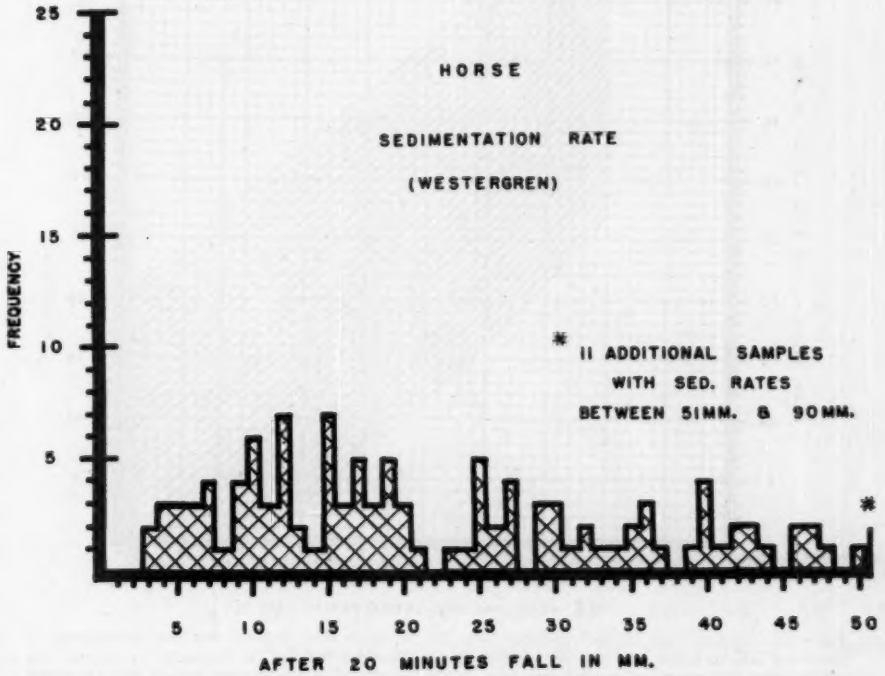

Fig. 3—Most of the samples are in the phase of maximal sedimentation velocity. The range in sedimentation rate is 3 to 90 mm.; the average is 25.1 mm.

into the phase of maximal sedimentation velocity. The average fall was 13.7 mm., while the range was from 2 to 47 mm. When these samples were examined at the end of twenty minutes, it was found that most of the samples were in the phase of maximal sedimentation velocity. From an examination of fig. 3, it may be seen that a range of 3 to 90 mm. existed and the average was 25.1 mm.

It was also noted that (1) the values of seemingly normal horses varied considerably, (2) there was considerable variation exhibited by any one horse over a period, (3) the time of maximal fall varied considerably, and (4) the sedimentation rate at the end of ten minutes appeared to be the only one showing data of possible statistical significance.

Within the various species, especially in man, where this phenomenon has been well studied, it has long been recognized that variations in type and amount of anticoagulant used, in temperature at which sedimentation occurs, and in width of tube used will cause variations in the sedimentation rate. Delay in setting up the test yields abnormally low values, as does inadequate aëration of the blood. Still of more importance is the fact that variation in the number of cells will affect that rate of fall, since it is known that cells and cell aggregates will fall faster in blood samples with few cells than in samples with normal or increased counts. For this reason, a correction is made for the degree of anemia and is usually based on hematocrit readings.[4] From such a correction, an increase in sedimentation rate resulting from reduction in red blood cells can be differentiated from a true increase owing to other alterations in the blood.

Van Zijl[3] found that a correction chart could be constructed for the horse if first

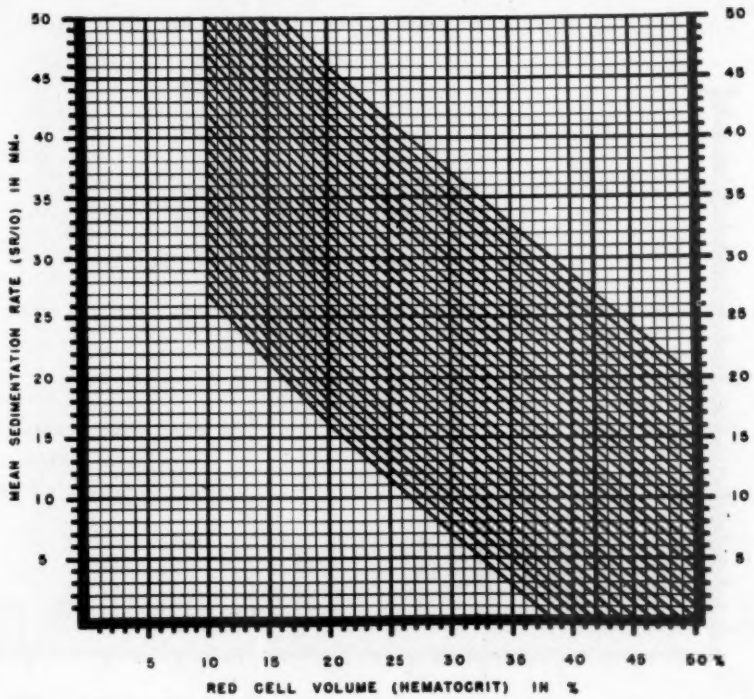

Fig. 4—Correction chart for sedimentation rate. Use of chart: First, find the line that corresponds to the mean sedimentation rate per ten minutes. Then, follow this line until it intersects the vertical line representing the volume of packed red cells. At this point, follow the nearest curved line downward until it intersects the heavy line at 42 per cent of the red cell volume. This point of intersection represents the corrected sedimentation rate, when read on the horizontal line.

the SR/10 were determined. The formula for the SR/10 was so devised that the first part of the sedimentation rate in the horse was emphasized and the high value of the sedimentation rate was reduced. However, his correction chart for blood of the horse was based on a normal red cell volume of 30 per cent, and this was considerably lower than the normal red cell volume of both the horses under investigation and the normals given for the breeds found in this country.[7, 8] Furthermore, the format of the chart was such that it was very difficult to interpolate a corrected value.

The SR/10 was determined for the 124 samples referred to earlier in this paper. A correction chart, patterned after the one prepared by Wintrobe and Landsberg[4] for human blood samples, but based on the SR/10 values, was prepared. With this chart (fig. 4), the sedimentation rate was corrected to a red cell volume of 42 per cent, thus removing one very important variable, namely, variation in red cell volume. The corrected SR/10 of the 124 samples previously discussed was found to average 15.04 mm., with a range of 7 to 23 mm. embracing 97 per cent of the samples. (*See* fig. 5.)

From data published elsewhere,[9] horses experimentally infected with equine infectious anemia have shown marked increases in their sedimentation rate after ten minutes' fall, when comparing the normal uninfected period with the rate exhibited on the tenth to fifteenth days of infection. These increases paralleled the drops described for the red cell counts, the hemoglobin, and the red cell volume. However, when the SR/10 was calculated and corrected to a red cell volume of 42 per cent, it was found that the corrected sedimentation rates showed no significant change during the course of an acute attack. Horse 24, for example, showed sedimentation rates of 18, 8, 45, and 86 mm. after ten minutes' fall on the fifth, seventh, twelfth, and fifteenth days of infection, re-

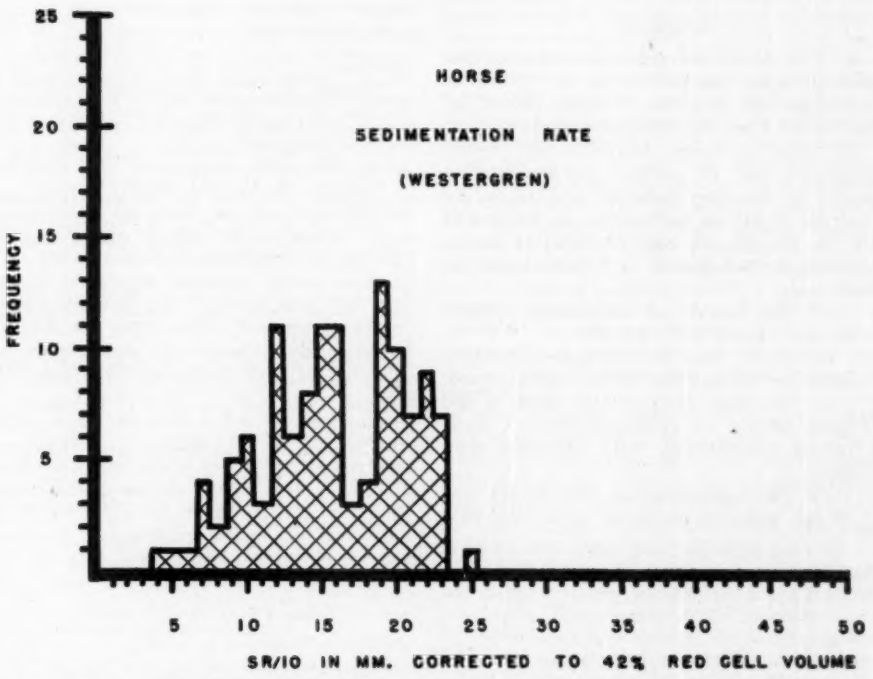

Fig. 5—The corrected mean sedimentation rate per ten minutes of the 124 samples averages 15.04 mm., with a range of 7 to 23 mm. embracing 97 per cent of the samples.

spectively. However, the SR/10 corrected to a red cell volume of 42 per cent for the same days became 21, 20, 20, and 20 mm., respectively. It may be concluded from this that in equine infectious anemia the changes in sedimentation rate observed were merely the result of changes in red cell volume.

As to the true nature of rapid sedimentation, it is presently thought to be due to alterations in the chemical constituents of the plasma and especially to increases in fibrinogen and globulin, which cause the cells to stick to each other in rouleaux formation.

Parker[10] sums up the value of the test by saying ". . . because the sedimentation rate is subject to many technical errors and represents the composite effect of numerous variables, it is obvious that too much attention should not be paid to minor variations nor too much weight given results. One of the principle errors in this procedure has been the error on the part of the clinician to give to the sedimentation rate a specificity which it does not deserve."

SUMMARY

1) The blood sedimentation rate of the normal horse was studied in the course of investigations into the changes caused by the virus of equine infectious anemia.

2) After running 124 different determinations on 13 normal horses over a period of thirteen months, and recording the rate of fall in millimeters at the end of 10, 15, 20, 30, 45, and 60 minutes to determine normal ranges, it became apparent that:

a) The values of seemingly normal horses varied considerably.

b) There was considerable variation exhibited by any one horse over a period.

c) The time required for each of the three phases of sedimentation to occur varied considerably with different samples.

d) The sedimentation rate at the end of ten minutes appeared to be the only one showing data of possible statistical significance.

3) A correction chart, based on the mean sedimentation rate per ten minutes (SR/10) is presented. With this chart, the sedimentation rate is corrected to a red cell volume of 42 per cent, thus removing one very important variable, namely, variation in red cell volume. The corrected SR/10 of the 124 normal samples investigated averaged 15.04 mm., with a range of 7 to 23 mm. embracing 97 per cent of the samples.

4) The enormous changes in sedimentation rates observed in equine infectious anemia were found to be the result of changes in red cell volume. When these rates were corrected to a red cell volume of 42 per cent, it was noted that no significant change in sedimentation rate existed during any stage of the disease.

5) The interpretation of the results of the sedimentation rate should be made with caution, because the rate is subject to both technical errors and numerous variables. Too much attention should not be paid to minor variations nor too much weight given to results.

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Plasma Protein Changes in Equine Infectious Anemia

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IN THE COURSE of investigating the changes brought about in the horse in response to the virus of equine infectious anemia, systematic serial studies of the plasma proteins were made on experimentally infected acute, subacute, and chronic carrier horses. This study was indicated in order to throw some light on such phenomena of clinical interest as edema formation, rapid sedimentation rate, and the elaboration of antibodies. This investigation has revealed significant quantitative changes in the concentration and fractional distribution of various plasma proteins during the course of this disease.

Systematic surveys of the plasma proteins in a great variety of disorders of human beings indicate that many diseases are not accompanied by any significant alteration in the plasma protein pattern. However, of the 16 disorders in which significant alterations do occur, five of these show disturbances in plasma proteins so marked or characteristic as to be somewhat pathognomonic.[1] No such information is available concerning changes in the distribution of the plasma or serum components in the disorders of the horse. For one thing, some of the simpler procedures evolved in human medicine were designed for human serums which differ considerably from horse serums, both quantitatively and qualitatively.[2, 3] Furthermore, it is important to appreciate that the various methods employed yield only crude fractional separations, which are reproducible when applied by standardized technique and, though helpful, do not satisfactorily resolve the plasma proteins into anything more than heterogeneous fractions satisfying certain conventional criteria. As a result, there are numerous discrepancies in both estimation and characterization by the several methods employed. However, better insight into both the significance and limitations of the data is now available as a result of recent refinements of technique. Gutman[4] offers an excellent appraisal and summary of the more significant data available both from the standpoint of methods and interpretation of results for human serums.

It is the purpose of this paper to point out the significant quantitative changes in the concentration of the various serum proteins during the course of this disease, correlating these findings with the clinical picture and pathological changes so that a fair understanding may be gained as to the disease processes involved.

METHODS

Total Serum Protein, Albumin, Globulin, and A/G Ratio.—Total Serum Protein.—Total serum protein was determined using a modified Greenberg[5] procedure. This involved making a 1 : 10 dilution of serum with physiological saline. The diluted serum (2.0 ml.) was placed in a 100.0-ml. volumetric flask. Twenty milliliters of distilled water was added and then 4.0 ml. of 10 per cent sodium hydroxide (w/v). Next, 3.0 ml. of phenol reagent (Folin and Ciocalteu) was added drop by drop with constant agitation. The volumetric flask was diluted to volume with distilled water and allowed to stand exactly ten minutes to develop the color. It was read, using the green filter (530 μ) with distilled water as a blank on the Klett-Summerson photoelectric colorimeter. The colorimeter was previously standardized by setting up 100.0-ml. flasks containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg. of tyrosine, respectively, and running as outlined above. A linear relationship existed, calibrated in terms of milligrams of tyrosine per 100 ml. of serum. The grams of total protein per 100 ml. equaled the milligrams of tyrosine per 100 ml. of serum times 8.

Serum Albumin.—Sodium sulfate (26%) was used to separate the serum albumin, the values being in close agreement with both electrophoresis and methanol partition procedures.[6, 7] This procedure involved placing 9.5 ml. of 27.4 per cent sodium sulfate, which had been kept at 37 C., into a test tube containing 0.5 ml. of serum. The tube was stoppered, inverted, and left in the incubator at 37 C. for a minimum of two hours. The contents of this tube were then filtered through a Whatman No. 50 filter paper in the incubator until a clear filtrate was obtained. Five milliliters of this filtrate was placed in a 100.0-ml. volumetric flask. The rest

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of the procedure was the same as for the total protein determination described previously, starting with the addition of 20.0 ml. of distilled water. The milligrams of tyrosine per 100 ml. of serum times 6.64 equaled the grams of albumin per 100 ml. of serum.

Serum Globulin and Albumin/Globulin Ratio.—The total serum protein and the serum albumin were determined as described previously. The serum globulin was determined by difference. The albumin/globulin (A/G) ratio was calculated by the following: $\frac{\text{albumin}}{\text{globulin}}$

Thymol Turbidity Test.—Six milliliters of thymol-barbital buffer, pH 7.8, were added to 0.1 ml. of serum in a colorimeter tube. The contents were shaken and allowed to stand for thirty minutes. The tube was inverted once and read in a Klett-Summerson photoelectric colorimeter, using the red filter (640 to 700 μ) against a blank containing 6.0 ml. of the thymol-barbital buffer and the galvanometer adjusted to zero.[8] The turbidity was expressed in units derived from a curve prepared from barium sulfate suspensions.[9]

Cephalin-Cholesterol Flocculation Test.—Serum (0.1 ml.) was added to 1.58 ml. of alkaline 0.85 per cent saline to effect the proper serum dilution.[10] Of this serum-saline mixture, 0.1 ml. was transferred to a 150 by 15 mm. test tube, and 0.5 ml. of cephalin-cholesterol emulsion[11] and 6.0 ml. of alkaline 0.85 per cent saline were added. The tubes were carefully shaken, stoppered with cotton, and placed in the dark for forty-eight hours at a temperature of 20 to 24 C. A "0" standard was made at the same time, corresponding to the same amount of antigen used in the test, while the other fluids were substituted with 0.85 per cent saline in a quantity equal to the quantities used in the test. Serial dilutions were made as described by Kibrick and Clements.[12] Interpretation and significance of the results were amply described in a recent modification of this test which is suitable for horse serums.

Blood Sedimentation Rate.—Whole blood samples were collected by puncture of the jugular vein into a tube containing a dried film of ammonium and potassium oxalate, as described by Heller and Paul.[13] Westergren pipettes were used. The rate of fall was recorded at the end of thirty and sixty minutes. All determinations were run within one and one-half hours of the time of collection, and a temperature of 20 to 25 C. was maintained throughout this procedure. The mean sedimentation rate per ten minutes (SR/10) was calculated and corrected to a red cell volume of 42 per cent, as described in a previous paper.[14]

RESULTS

Total Serum Protein, Albumin, Globulin, and Albumin/Globulin Ratio.—From the accumulated data, it appears that there was a slight rise in total serum protein following the experimental infection of the horse with the virus of equine infectious anemia when an acute case resulted. This was accompanied by a great drop in the serum albumin and a rise in the serum globulin, resulting in extremely low A/G ratios. This pattern was presented in each experimentally infected acute case to a greater or lesser degree, depending upon the severity of the attacks and the number of such attacks the animal might go through before it either terminated in death or recovered to become a chronic carrier.

Table 1 presents a typical experimentally infected acute case of equine infectious anemia (horse 22). The first attack started on the tenth day of experimental infection, when the temperature reached 104.2 F., and lasted through the fifteenth day. From the sixteenth until the twenty-sixth day, the highest temperature recorded was 101.4 F., yet there was a tremendous drop in serum

TABLE 1—Acute Case of Equine Infectious Anemia, Horse 22*

Day of infection	Serum total protein	Serum albumin	Serum globulin	A/G ratio	Cephalin-cholesterol flocculation	Thymol turbidity test
	(Gm. per 100 ml. of serum)					
Normal (control period)	7.64	2.92	4.72	.62	Negative	2.0 units
10th	7.26	2.58	4.68	.55	Negative	1.5 units
12th	7.12	1.99	5.13	.39	Negative	2.0 units
15th	7.36	1.99	5.37	.37	Negative	2.5 units
17th	7.04	.90	6.14	.15	Negative	2.3 units
24th	7.92	1.59	6.33	.25	Positive	2.8 units
29th	7.52	.69	6.83	.10	Positive	2.8 units
34th	7.84	1.33	6.51	.20	Positive	2.8 units
36th	7.84	2.16	5.68	.38	Positive	1.7 units
41st	6.64	1.20	5.44	.22	Positive	4.2 units
50th	7.68	1.82	5.86	.31	Positive	9.1 units
59th	8.64	1.74	6.90	.25	Positive	11.1 units

*Reaction No. 1, between tenth and fifteenth days. Reaction No. 2, between twenty-sixth and twenty-ninth days. Reaction No. 3, between thirty-fifth and fifty-ninth days.

albumin on the seventeenth day, a rise in the serum globulin level, and a resultant lowering of the A/G ratio. From the fifteenth to the seventeenth day, there was a drop of 55 per cent in serum albumin, while there was a gain of 14.3 per cent in serum globulin. The total serum protein showed a drop of less than 5 per cent for this same period. In two days (from 17th to 19th), there was a rise in all components but, at the same time, it may be noted that the serum globulin was above normal,* the serum albumin was below normal,* and the A/G ratio was still abnormally low.* The second attack occurred during the period between the twenty-sixth and twenty-ninth days. Again on the twenty-ninth day, we had the same cycle repeating itself. This animal then had a third attack, with a continuous temperature of over 102 F. from the thirty-fifth day until it was destroyed on the fifty-ninth day. One may note that the low A/G ratio continued to be a feature.

Gm. per 100 ml. of serum, which was 57 per cent of the low normal of 2.3 Gm. per 100 ml. of serum. The globulin averaged 7.09 Gm. per 100 ml. of serum for this same period, and this was 139 per cent higher than the high normal for the horse. The A/G ratio was likewise altered, being 65 per cent below the low normal value.

One chronic carrier animal was available for study for sixteen months. During this period, four attacks were noted. In the interim between attacks, the serum total protein, albumin, globulin, and A/G ratio values all were found to lie in the normal range. However, in the periods of attack, these values departed from the normal range, demonstrating the same trends as the acute cases described, but not as severe.

Cephalin-Cholesterol Flocculation Test.—The serial studies of serum samples taken from 7 acute cases of equine infectious anemia showed that, on the average, seventeen days passed from the date of experimental infection until the animal demonstrated a positive cephalin-cholesterol flocculation test. Two animals became positive on the eleventh day, 1 on the fifteenth, 1 on the nineteenth, and 3 on the twenty-first day. The apparent discrepancy in time necessary to secure a positive test appears to be dependent upon the length of the incubation period and the severity of the first attack. The longer the incubation period, the longer it will take to demonstrate the first positive test. Furthermore, the more acute the attack, the sooner will this test turn positive. Conversely, with a mild first attack, there may not be sufficient insult to the tissues to evoke a positive test.

TABLE 2—Acute Case of Equine Infectious Anemia, Horse 21*

Day of infection	Serum total protein	Serum albumin	Serum globulin	A/G ratio	Cephalin-cholesterol flocculation	Thymol turbidity test
	(Gm. per 100 ml. of serum)					
Normal (control period)	7.99	3.07	4.92	.62	Negative	0.7 units
46th	7.55	1.73	5.82	.30	Positive	1.9 units
54th	7.71	1.62	6.09	.27	Positive	2.7 units
70th	8.40	1.14	7.26	.16	Positive	9.2 units
78th	8.45	1.24	7.21	.17	Positive	8.0 units
85th	8.61	1.35	7.26	.19	Positive	6.3 units
92nd	9.04	1.46	7.58	.19	Positive	5.7 units
102nd	7.96	1.20	6.76	.18	Positive	6.0 units
109th	8.22	1.55	6.67	.22	Positive	5.1 units
117th	8.91	1.25	6.66	.19	Positive	4.4 units
126th	8.75	1.40	7.35	.19	Positive	10.5 units

*Reaction No. 1, between seventh and ninth days. Reaction No. 2, between thirty-third and thirty-eighth days. Reaction No. 3, between forty-third and sixty-third days. Reaction No. 4, between 113th and 139th days.

Table 2 presents another rather acute case (horse 21). Although its first reaction was quite weak, the subsequent reactions were much stronger and of longer duration (3rd reaction 20 days in length, while the 4th lasted 26 days). It is interesting to note that in the interim between the third and fourth reaction (a period of 48 days), the high total serum protein, low serum albumin, high serum globulin, and very low A/G ratio continued to be a striking feature. From the seventieth until the 126th day, the albumin averaged 1.32

From table 3, it may be noted that horse

*Normals: total serum protein, 6.3 to 8.6 Gm. per 100 ml. of serum; serum albumin, 2.3 to 3.5 Gm. per 100 ml.; serum globulin, 4.0 to 5.1 Gm. per 100 ml.; A/G ratio, 0.55 to 1.0. From preliminary studies in this laboratory on normal mature horses.

25 was followed for sixty days after it showed its first positive test, that 11 serum samples from this horse were examined over this period, and that all these serum samples were positive. It may also be noted that, of the 46 serum samples examined from these animals subsequent to the time they became positive to this test, all 46 were positive.

TABLE 3—Results of the Cephalin-Cholesterol Flocculation Test with 11 Cases of Equine Infectious Anemia

Horse (No.)	Control period	Day of infection first positive	No. serum samples examined after first positive	No. samples positive	Day of infection destroyed
13	3 months	21st day	5	5	56th day
22	1 day	19th day	9	9	59th day
24	5 months	15th day	1	1	15th day
25	3 months	21st day	11	11	81st day
27	8 months	21st day	6	6	49th day
29	1 day	11th day	3	3	18th day
30	4 months	11th day	11	11	54th day
21	*		44	40*	139th day
31	7 months	**	21	21	152nd day
32	4 months	***	..	..	.....
26	1 month	****	..	..	10th day

*First samples tested were after vaccination. Twenty samples, starting with the eleventh day after challenge, were all positive. Of the 24 previous samples, only four were negative. **Challenged twenty-seven days after vaccination. Became positive two days after challenge. ***Weak reaction to attenuated virus. Became a carrier; destroyed. ****Destroyed on tenth day after challenge.

Two vaccinated animals presented a somewhat different picture. Both received repeated large doses of material, were proved noninfected after vaccination, and were successfully challenged. It appears that both of these animals gave a positive cephalin-cholesterol flocculation test, 1 horse two days after challenge (29th day after last vaccination) and the other for one year after vaccination. During this one-year period, 24 serum samples were examined and 20 of these were positive. However, both of these animals were consistently positive after being challenged.

One subacute case (horse 32) reacted quite weakly to attenuated virus after an incubation period of nineteen days. Although four temperature excursions were charted, the reactions were considered minor from the standpoint of blood changes and A/G ratio changes. This horse never became positive to the cephalin-cholesterol flocculation test and was destroyed because it had become a chronic carrier.

One acute case (horse 26), which was destroyed on the date of its first temperature rise (10th day after experimental infection), was negative to the test at that time.

One chronic carrier animal (horse 10) was available for study sixteen months. This animal demonstrated a positive cephalin-cholesterol flocculation test for all 42 of the nearly weekly samples taken over this sixteen-month period.

Thymol Turbidity Test.—The thymol turbidity test was applied to all serial serum samples taken from the horses used in this study. If 0.0 to 4.0 units were taken as the normal range for the horse,* then 9 acutely infected animals followed 10, 15, 18, 36, 49, 54, 56, 81, and 122 days, respectively, after experimental infection remained negative to this test. All of these animals reacted acutely between the tenth and the fourteenth days of experimental infection and exhibited both high fever and severe blood changes. However, 2 other animals similarly infected did become positive to this test. Horse 22 became positive on the forty-first day and remained so until destroyed on the fifty-ninth day (*see* table 1). The values of the four samples examined during this period were 4.2, 6.9, 9.1, and 11.1 units, respectively. In the case of horse 21, a positive test was demonstrated on the seventieth day, remaining positive until destroyed on the 139th day (*see* table 2). Serum samples examined during this period revealed values of 9.2, 8.0, 6.3, 5.7, 6.0, 5.1, 4.4, and 10.5 units.

TABLE 4—Sedimentation Rate of an Acute Case of Equine Infectious Anemia

	Control period	5th day	7th day	12th day	15th day
10-minute fall in mm.	13	18	8	45	86
15-minute fall in mm.	31	43	27	106	116
20-minute fall in mm.	61	83	53	122	122
SR/10* corrected	21	21	20	20	20

*Mean sedimentation rate per ten minutes.

However, both of these animals were extremely acute cases. One animal ran a continuous temperature above 102.5 F. for twenty-three days, while the other one did

*Preliminary studies from 135 normal horses demonstrated that 0.0 to 4.0 units covered 100 per cent of the samples examined.

the same thing for twenty and again for twenty-six days.

One chronic carrier animal (horse 10) was followed almost weekly for sixteen months. During this time, despite four attacks, every sample examined was negative. From these data, one must conclude that a positive thymol turbidity test will only occur in the extremely acute case which has had repeated attacks.

Blood Sedimentation Rate.—The sedimentation rate of the red blood cells was carefully followed in 10 acute cases of equine infectious anemia, both during the control period and after experimental infection. It was noted that there was a marked increase in the sedimentation rate after ten minutes' fall, when comparing the normal control period with the rate exhibited on the tenth to fourteenth days of infection. These increases paralleled the drops described for the red cell counts, the hemoglobin, and the red cell volume.[15] However, when the SR/10 was calculated and corrected to a red cell volume of 42 per cent, it was found that the corrected sedimentation rates showed no significant change during the course of the disease. From table 4, it may be noted that, in the case of horse 24, the sedimentation rate per ten minutes' fall during the control period of two months averaged 13 mm. On the fifth, seventh, twelfth, and fifteenth days of infection, the sedimentation rates were 18, 8, 45, and 86 mm., respectively. However, the SR/10 corrected to a red cell volume of 42 per cent for the control period became 21 mm., while the values on the other days became 21, 20, 20, and 20 mm., respectively. Essentially, the same results were noted in the other 9 cases.

One chronic carrier animal (horse 10) was followed almost weekly for sixteen months. During this time, the sedimentation rate per ten minutes' fall averaged 5 mm., with the extremes being 1 and 15 mm. The values for the SR/10 corrected to a red cell volume of 42 per cent were within the normal limits of 7 to 23 mm.[14]

DISCUSSION

In pathological conditions, changes in the distribution of protein components may occur. When they do take place, the abnormalities follow a general pattern. The highest plasma albumin levels occur in healthy subjects (excluding hemoconcentration in dehydration), and the effect of disease, particularly of disorders associated with marked wasting or malnutrition, invariably is lowering of the serum albumin level. This hypoalbuminemia is especially pronounced where there is a marked loss of albumin in the urine (*i.e.*, nephritis) or where there is a disturbance in albumin formation (*i.e.*, cirrhosis of the liver). The effect of various diseases on the total serum globulin fraction, on the other hand, is to cause hyperglobulinemia, which may be very marked in certain infections and liver disorders. Yet, hyperglobulinemia is often associated with hypoalbuminemia, so that the level of the total serum proteins may be within normal limits despite the changes in both the albumin and globulin levels. Howe,[16] in 1921, introduced the use of 21.5 per cent sodium sulfate for separation of serum albumin from serum globulin and, until very recently, this was the method of choice because of its simplicity. Tiselius[17] applied the newly perfected electrophoresis technique to various serums and identified the various components according to their different electrophoretic mobility. He reported that the so-called pseudoglobulin and euglobulin fractions represented mixtures of several globulin components and that they differed chiefly in their solubility behavior rather than in different composition. Then, other workers,[6,7,12] using this same method, showed that the albumin fraction of Howe actually contained considerable amounts of α and β globulin, and that A/G ratios, as we know them, were actually too high. For this reason, methods were chosen which yielded results in close agreement with electrophoresis.

In order to gain as complete a picture of the various plasma protein changes, if any were to exist, both indirect and quantitative methods were used, upon which specific information was available as to the mechanism of action. For this reason, the cephalin-cholesterol flocculation test was applied. It is now known that a positive flocculation will occur[18] when (1) there is a rise in γ globulin, (2) albumin falls below the amount necessary to inhibit the globulin, or (3) there is a reduction in the inhibitory fraction of the serum albumin. Thus, a positive test can be expected in primary hepatic impairment because the liver is the site of albumin production. It is likewise to be expected that in conditions

in which the reticulo-endothelial system is involved without primary hepatic impairment (*i.e.*, malarial fever), a positive test will result due to stimulation of γ globulin from that site.[19]

The thymol turbidity test was selected because this reaction depends upon elevated α and β globulins as well as lipids, resulting in the precipitation of a globulin-thymol-lipid complex.[20,21] The elevation of the sedimentation rate is believed to be related to increased plasma fibrinogen[22] and α globulin.[23] Since it is now generally accepted that the liver is the site of production of fibrinogen, albumin, and the globulin fractions with the exception of γ globulin,[4] the above determinations may be used as a cross section in estimating the functional capacity of the liver.

From the data which has been presented, it is to be noted that the most striking feature has been the elevation of the cephalin-cholesterol flocculation test. This in itself is not of such a singular nature, but when the amounts of serum albumin are available for evaluating these positive tests, it is to be noted that positive cephalin-cholesterol tests occur despite wide fluctuations in the serum albumin. This presumably points to the reticulo-endothelial system as a primary target for the virus. Further evidence to support this view comes from Ehrich *et al.*,[24] who recently demonstrated that the γ globulin production is a function of the plasma cells which are most plentiful in the lymphoid elements of the reticulo-endothelial system.

From the purely functional aspect, there is the likelihood that the liver is not too badly involved. This is indicated by very little change, if any, in the thymol turbidity tests and the sedimentation rates. However, it must be assumed that impairment does exist as a result of the extreme low values of serum albumin which have been recorded, since urinary loss alone could not account for this. The lowest serum albumin levels were usually found some days after the temperature had returned to normal. In still other cases, attacks have taken place without a temperature rise but have demonstrated the characteristic blood changes and A/G ratio changes. This would indicate the inability of the liver to manufacture serum albumin during these periods.

The histological changes described by Fedorov[25] for equine infectious anemia included a marked activation of the cells of the reticulo-endothelial system in the liver, kidney, spleen, and myocardium. The proliferative foci were composed of histiocytes and lymphoid elements. This is further evidence of the involvement of the reticulo-endothelial system.

As far as this disease is concerned, the A/G ratio is the most sensitive of the liver function tests applied in this study. This test follows the pattern of attacks with regularity and is a good guide to the clinical condition of the animal and the severity of the attack. The cephalin-cholesterol flocculation test is the best indication that the disease process is still actively at work, despite the apparent recovery which characterizes the chronic carrier. Lastly, the thymol turbidity test is an indication of an extremely severe case.

SUMMARY

1) Systematic serial studies of the plasma proteins were made on experimentally infected acute, subacute, and chronic cases of equine infectious anemia. Specifically, data were charted on serum total protein, serum albumin, serum globulin, albumin/globulin (A/G) ratios, the cephalin-cholesterol flocculation test, thymol turbidity test, and sedimentation rates.

2) It appeared that there was a slight absolute rise in total serum protein following experimental infection of the horse with equine infectious anemia virus when an acute case resulted. This was accompanied by a great drop in the serum albumin and a rise in the serum globulin, resulting in extremely low A/G ratios.

3) This pattern was presented to a greater or lesser degree, depending upon the severity of the attack and the number of such attacks the animal might go through before it terminated in death or recovered to become a chronic carrier.

4) The cephalin-cholesterol flocculation test was found to become positive in the acute cases studied, as a rule between the first and second attacks. However, where the first attack results in a subacute case which soon becomes a chronic carrier, there may not be sufficient insult to the tissues to produce a positive cephalin-cholesterol flocculation test. Such a situation existed in only 1 out of 11 cases reported. The other 10, after once becoming positive, re-

mained positive for as long as these animals were studied (up to 16 months with 1 chronic case).

5) The thymol turbidity test became positive only in an extremely acute case which had had three or more severe attacks. Nine out of 11 cases examined remained negative to this test.

6) The sedimentation rate of the red blood cells showed no significant change during the course of this disease when the mean sedimentation rate per ten minutes was calculated and corrected to a red cell volume of 42 per cent. The marked apparent increases in rates observed were actually due to lowering the red cell volume.

7) The reticulo-endothelial system appeared to be an important target for the virus of equine infectious anemia in the horse.

8) From the liver-function tests applied in this study, the following conclusions may be drawn: the A/G ratio was the most sensitive to changes during the course of this disease; the cephalin-cholesterol flocculation test was the best indication of the chronicity of the disease; while the thymol turbidity test usually indicated an extremely severe case.

9) Although significant alterations in both the serum proteins and certain liver-function tests occurred in equine infectious anemia and appeared to be regular features of the disease, it does not imply that any of these changes were specific for this disease.

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The Time of Closure of the Lower Femoral Epiphyses and Upper Tibial Epiphyses in the Dog as Determined by Roentgenogram

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BECAUSE OF THE necessity of knowing the approximate time of closure of the lower femoral epiphyses and upper tibial epiphyses in the dog in carrying out bone growth experiments in that animal,[1] we selected 8 dogs of known date of birth and pet and was kept in the home of one of us (Janes). It received a diet consisting of a commercially prepared dog food* supplemented with table scraps.

All of the dogs used in this experiment were given distemper prophylaxis and were made roentgenograms of the knee region at regular intervals. Table 1 illustrates the results.

TABLE 1—Dates of Closure of Epiphyses

Dog	Date of birth, 1949	Sex	Breed	Condition of epiphyses at given dates										Closed (months)
				12-9-49	1-4-50	2-2-50	3-2-50	4-4-50	5-2-50	6-2-50	7-5-50	8-2-50	9-2-50	
1	9-1	F	Mongrel terrier type	Open	Open	Open	Open	Open	Open	Closing	Closed	.....	.....	10
2	9-1	F	Mongrel terrier type	Open	Open	Open	Open	Open	Open	Closing	Closed	.....	.....	10
3	9-1	F	Mongrel terrier type	Open	Open	Open	Open	Open	Open	Closing	Closed	.....	.....	10
4	10-11	F	Collie and Labrador	Open	Open	Open	Open	Open	Open	Closing	Closing	Closed	.....	10–11
5	10-11	F	Collie and Labrador	Open	Open	Open	Open	Open	Open	Open	Closing	Closing	Closed	11
6	10-11	F	Collie and Labrador	Open	Open	Open	Open	Open	Open	Open	Closing	Closed	.....	10–11
7	10-11	F	Collie and Labrador	Open	Open	Open	Open	Open	Open	Closing	Closing	Closed	.....	10–11
8	7-22	M	Boxer	.....	Open	.....	Open	Open	.....	.....	Closed	.....	.....	11–12*

*If a roentgenogram of this dog had been made at the tenth month, it might have shown that the epiphyses had closed by that time.

Dogs 1 through 7 were laboratory animals. They were confined in the laboratory kennels in pens which were large enough to permit them to exercise freely. The daily diet of these dogs consisted of a commercially prepared dog food and a cooked food prepared by us, consisting of corn meal, oatmeal, wheat middlings, dried meat scraps, salt, and water. Dog 8 was a family treated for parasites. They remained in good health and made normal growth.

CONCLUSIONS

From this study of 8 dogs, it appears that according to roentgenograms the lower femoral epiphyses and the upper tibial epiphyses close at about the age of 10 months. Accordingly, it would seem that any attempt to stimulate bone growth in the hind extremities of dogs must be made sometime before the tenth month.

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*Friskies, manufactured by Albers Milling Company, Peoria, Ill.

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Experimental Q Fever in Cats

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IN MANY REPORTS concerning Q fever, efforts have been directed toward finding an epidemiological relationship between infection in human beings and the various reservoirs of *Coxiella burnetii* that have been found in domestic and wild animals and various ectoparasites.[1-7] In one area in California, it appeared that the consumption of contaminated raw milk from cows whose udders harbored organisms or contact with infected livestock was responsible for the infection.[7] However, in another area in California, the drinking of contaminated raw milk or contact with livestock was not wholly incriminated, and the route of infection seemed to be nasal.[6] Some workers think that the immediate source of infection in man is air contaminated by infected animals[6] but that the infection is not spread directly between people, even though they eliminate organisms in sputum and urine.[6,8] As yet, in spite of considerable work, it seems that there remain sources and means of transmission not entirely known.

While studying experimentally induced infection in calves with *C. burnetii*, cats were tested for susceptibility. Cats were chosen since, by their habits, they might bring infection from natural sources to contaminate the air of man's environment. Accordingly, experimental work was initiated. The findings are herewith presented.

MATERIALS AND METHODS

Production of Infection.—The strain of *C. burnetii** used in these studies was obtained from the National Institutes of Health, Bethesda, Md., in the form of infected yolk sacs of hens' eggs, and had been isolated in California from the milk of a naturally infected cow. Suspensions of organisms for experimental use were prepared by inoculating guinea pigs intraperitoneally with 1 cc. of 10^{-1} dilution of infected yolk sac and removing their spleens on the fifth day after inoculation. The spleens were weighed and made into a 10 per cent suspension with sterile skimmilk in a glass grinder (TenBroeck type). In all the egg work that follows, chicken eggs that had been embryonated for six days were used, and all eggs were inoculated by the yolk sac route. Suspensions of yolk sacs were prepared by inoculating other eggs with 0.5 cc. of a 10^{-1} suspension of infected yolk sac. The eggs, after incubation at 37 C. for six days, were harvested when the embryos became moribund. All suspensions were distributed in stoppered vials, frozen with a mixture of dry ice and alcohol, and stored under dry ice refrigeration. Usual aërobic and anaërobic examinations of these suspensions for bacteria showed no growth. Titrations for concentration of *C. burnetii* were made by inoculating groups of five eggs each with the 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} dilutions. Each egg was given 0.5 cc. of the respective dilution and incubated at 37 C. for twelve days. On the basis of embryo mortality, an end point of lethal dose$_{50}$ (l.d.$_{50}$) $10^{-9.2}$ was found by the method of Reed and Muench.[10]

Cats were procured from farms and placed in separate cages, except in instances where contact exposure was planned. These animals varied in age from 3 to 15 months. Cats usually were fed a mixture of horse meat and powdered milk. Whenever fresh cow's milk was fed, it was heated to more than 90 C. for five minutes, a temperature deemed adequate to destroy any organism pathogenic for cats. Prior to use, temperatures and observations were made daily for at least one week. Only 1 cat showed an unplanned infection.

In attempts to produce infection, 4 cats were each inoculated subcutaneously with 1 cc. of either yolk sac suspension (No. 1, 3, and 4) or guinea pig spleen suspension (No. 2); 3 cats were each fed 1 cc. of infected yolk sac (No. 5, 7, and 8); 1 cat (No. 6) was fed 1 cc. of guinea pig spleen suspension; and 4 cats (No. 9, 10, 11, and 12) were placed in cages with the cats inoculated subcutaneously.

Tests for *C. burnetii* consisted of intraperitoneal inoculations into guinea pigs with 1 cc. of either blood or urine that had been collected from the cats at periodic intervals. Blood was removed by cardiac puncture, and urine either by catheterization or direct puncture into the bladder through the abdominal wall. The criteria for the presence of *C. burnetii* in the blood and urine were pyrexia of the guinea pig, the presence of complement-fixing antibodies, and refractivity of the guinea pigs

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when later inoculated with a yolk sac suspension from infected eggs that caused signs of illness and occasionally death in control animals. The results are presented in table 1.

All cats (No. 1, 2, 3, and 4) inoculated subcutaneously, except cat 2, inoculated with the guinea pig spleen suspension, showed an increased temperature that began two days after inoculation and lasted for three days. During the febrile period, they also showed lethargy and lack of appetite. Cats that were fed either the guinea pig or yolk sac suspension or exposed showed no signs of illness.

Tests made on blood from cats 5, 7, and 8, fed yolk sac suspensions, showed the organism in the blood one week and two weeks after inoculation but not in later tests. In tests on urine, cats 5 and 7 showed the organism at the four-week interval; however, when cats 7 and 8 were tested at the eight-week interval, the organism was not isolated. A similar test on cat 6, fed guinea pig spleen suspension, showed no organisms in either blood or urine.

Following contact exposure, only cat 11 showed organisms in the blood three and four weeks afterwards, but cats 10 and 11, tested at the eight-week interval, were negative. Cat 11 showed organisms in the urine at the four-week interval, while cats 10 and 12 were negative. The urine of cats 10 and 11 was negative when tested at eight weeks.

TABLE 1—Presence of Coxiella Burnetii in Blood and Urine of Experimental Cats

Route of inoculation	Cat (No.)	Source of virus	No. weeks after inoculation: 0 B*	1 B	2 B	3 B	4 B	4 U**	8 B	8 U
Subcutaneous	1	Yolk sac	—	—	—	—	—	+	NT	NT
	2	Guinea pig spleen	—	+	—	—	—	NT	NT	NT
	3	Yolk sac	—	—	—	+	—	+	NT	NT
	4	Yolk sac	—	+	—	+	+	+	—	+
Oral	5	Yolk sac	—	+	+	—	—	+	NT	NT
	6	Guinea pig spleen	—	—	—	NT	—	—	NT	NT
	7	Yolk sac	—	+	+	—	—	+	—	—
	8	Yolk sac	—	+	+	—	—	—	—	—
Cage contact	9	.....	—	—	—	NT	—	NT	NT	NT
	10	.....	—	—	—	—	—	—	—	—
	11	.....	—	—	—	+	+	+	—	—
	12	.....	—	—	—	—	—	—	NT	NT

*Blood. **Urine. + = *coxiella burnetii* present. — = *coxiella burnetii* absent. NT = no test.

As can be seen in table 1, from the blood of cats 2, 3, and 4, inoculated subcutaneously, *C. burnetii* was isolated at various times. Cats 2 and 4 were positive in the first test made one week after inoculation, and the remaining cat 3 was positive three weeks after inoculation. *Coxiella burnetii* was present in cat 4 four weeks afterwards but not present when tested after eight weeks. Tests on urine were not as comprehensive as blood tests, but the organism was obtained from cats 1, 3, and 4 tested four weeks after inoculation and from cat 4 tested eight weeks afterwards.

Serological Studies.—Serums for the complement-fixation tests were obtained from all cats prior to inoculation, except cats 7 and 8, and again four and eight weeks afterwards. At the time of test, each serum was inactivated at 56 C. for thirty minutes. The antigen* used

*Through the courtesy of Dr. Herald Cox, Lederle Laboratories, Pearl River, N. Y.

TABLE 2—Serological Response of Experimental Cats Inoculated with Coxiella Burnetii

Route of inoculation	Cat (No.)	Infection	No. weeks after inoculation: Complement-fixing titer 0	4	8	Agglutination titer 0	4	8
Subcutaneous	1	Present	....	....	1:16	....	....	....
	2	Present	....	....	1:32	....	....	1:32
	3	Present	....	1:128	1:16	....	1:32	....
	4	Present	....	1:32	1:16	....	1:32	1:16
Oral	5	Present	....	....	....	....	....	1:8
	6	Absent	....	....	NT	....	....	NT
	7	Present	NT	1:16	1:16	....	1:8	1:16
	8	Present	NT	....	....	NT	....	....
Cage contact	9	Absent	....	....	NT	....	....	NT
	10	Absent	....	....	NT	....	....	NT
	11	Present	....	....	1:8	....	....	1:16
	12	Absent	....	....	....	....	....	....

NT = no test.

was supplied by Dr. Cox and was made with the Henzerling strain of *C. burnetii*. In the test, two units of complement, antigen, amboceptor, and a 2 per cent sheep red blood cell suspension, each in 0.5-cc. amounts, were used. In 0.5-cc. amounts, twofold dilutions of serum were tested for complement fixation. Adequate controls were used. The rickettsial system was maintained at 6 C. for eighteen to twenty-four hours prior to adding the sensitized red cell suspension. Complete fixation was used as the end point reading. Results are shown in table 2.

None of the serums obtained before inoculation or exposure fixed complement. Serums from cats 3 and 4 that had been inoculated subcutaneously showed complement-fixing antibodies four weeks afterwards, and serums from all cats that had been inoculated subcutaneously fixed complement eight weeks afterwards. Serum from cat 7, that had been fed, fixed complement at fours weeks and of cats 5, 7, and 8 tested at eight weeks, only cat 7 fixed complement. Of the group of 4 cats that had been exposed, none showed complement-fixing antibodies at four weeks and, of cats 11 and 12, tested at eight weeks, only cat 11 fixed complement.

The antigen used in the agglutination test was made with the same strain of *C. burnetii* used for cat-infectivity studies in this paper. The antigen was produced by the method of Shepard and Topping.[5] In the test, 0.2 cc. of rickettsial suspension of the desired turbidity were mixed with 0.2 cc. of suitable dilutions of antiserum and the mixture placed in a 5-mm. diameter tube. After incubation at 37 C. for four hours, the tests were placed in the refrigerator overnight and the results recorded the next morning. Complete agglutination was used as the end point reading. The results of the agglutination test are placed in table 2 for purposes of comparison with the complement-fixation tests.

No cats from which preinoculation serums were taken showed agglutinins before inoculation. Cats 3 and 4, inoculated subcutaneously, had agglutinins four weeks after inoculation, and cats 2 and 4 showed agglutinins eight weeks after inoculation. Serum from cat 7, that had been fed, agglutinated antigen four weeks afterwards, and, of cats 5, 7, and 8, tested at eight weeks, the serum of cats 5 and 7 agglutinated antigen. Cats 9, 10, 11, and 12, of the contact exposure group, were negative at four weeks and, of cats 11 and 12, studied at eight weeks, cat 11 had agglutinins. Tests with the other cats were negative.

DISCUSSION

A strain of *C. burnetii* was isolated from cow's milk. Cats were infected with this strain (which was grown in guinea pig spleen or the yolk sac of the embryonated chicken egg) by subcutaneous inoculation, by feeding, and by exposure to other cats. With the exception of cat 2, clinical infection occurred when cats were inoculated subcutaneously, while cats that were infected by feeding or exposure showed no signs of illness. *Coxiella burnetii* was found in the urine of experimentally infected cats for four and eight weeks after inoculation and appeared to constitute the source of infection to other cats that were exposed. The cat, by habit, has a wide range of activities that brings it into intimate contact with human beings and with many wild and domestic animals and their ectoparasites. If they were found to be infected naturally with Q fever and discharge *C. burnetii* in urine for long periods afterwards, cats could be important in the epidemiology of Q fever.

Either agglutinins or complement-fixing antibodies were present in serums of 7 infected cats, while the serum of 1 infected cat showed neither. This serological response compared favorably with isolations of *C. burnetii* in guinea pigs. These results suggest that the complement-fixation or agglutination test can be used to determine naturally occurring infection in cats where Q fever is endemic.

SUMMARY

Infection of cats with *Coxiella burnetii* has been produced by subcutaneous inoculation, by feeding, and by contact exposure. Most cats inoculated subcutaneously showed fever, lack of appetite, and lethargy that began two days after inoculation and lasted for three days, while animals infected by other routes showed no signs of illness. The organism was present in the blood of some cats for one month following inoculation and for two months in the urine. With 1 exception, complement-fixing antibodies or agglutinins were demonstrated in serums from infected cats. This ability of *C. burnetii* to infect cats suggests that they may be a factor in the spread of Q fever.

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Variations in Behavior of Two Strains of Newcastle Disease Virus on Passage Through Brains of Adult Mice

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TRANSMISSION OF Newcastle disease (ND) to adult mice by intracerebral inoculation is reported below, along with a description of its pathology in mouse brain. Two strains of Newcastle disease virus (NDV), the Victoria and the California 11914, were used, both having been adapted to growth in the brains of suckling mice as elsewhere described.[1] To our knowledge, ND has not been hitherto transmitted to adult mice by direct mouse adaptation. Several phenomena exhibited by the California strain 11914 of NDV appeared of interest. For one thing, it apparently developed sufficiently in adult mouse brain to cause clinical disease and pathological lesions, but failed to reach the infective stage of its developmental cycle. Secondly, after repeated passages in the brains of suckling mice, multiplication of this strain of NDV could not be demonstrated in embryonating eggs by any usual procedures.

METHODS

Methods employed for inoculations of mice and of eggs and for the antihemagglutination tests have been previously described.[1, 2] Normal and NDV-immune chicken serums used for identification of virus were kindly supplied by Dr. L. T. Giltner, of the Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

Results of Inoculations in Adult Mice.—Infected mouse brains in dilutions of 10^{-1} or 10^{-2} were used for intracerebral passage in mice from 3 to 4½ weeks old. Both strains of NDV led, in most instances, to a characteristic but not pathognomonic disease picture, after an incubation period ranging from two to eight or more days. Hyperexcitability was often the first sign of illness. Although the neurological manifestations varied, affected mice frequently had a jerking of the head to one side and a circling in one direction. Some would rub their forepaws together as they rose up on their hind legs, often falling over backwards. Such mice generally died within four to twenty-four hours, with unruffled fur. Occasionally, death was sudden following generalized convulsion. The Victoria was the more pathogenic of the two strains, being readily carried through ten consecutive passages in adult mice and producing, as about one half of the adult mice inoculated. On the other hand, attempts to pass the California strain 11914 from one adult mouse to another, or from adult mice back to suckling mice, were unsuccessful. This seemed surprising, as about one half of the adult mice inoculated intracerebrally with brains of suckling mice infected with the California strain developed a characteristic illness. A possible explanation of this failure of the virus to develop infection in adult mouse brain is offered below. Both strains of mouse-adapted NDV were infectious for suckling mice by the intraperitoneal route at a concentration of 10^{-1} but not at higher dilutions.

Identification of Virus by Neutralization Test.—After 22 passages in mice, infective agents were reidentified as NDV by means of neutralization tests in brains of suckling mice. Both NDV-immune and normal chicken serums were inactivated by heating at 56 C. for one-half hour in dilutions of 1 : 2 before mixing with equal amounts of tenfold dilutions of virus in buffered saline. Serum-virus mixtures were inoculated intracerebrally into 6-day-old mice in 0.03-cc. amounts, after standing at room temperature for about one-half hour. The standard NDV-immune serums completely neutralized all dilutions of both virus strains, whereas, in the presence of normal serum, the California strain 11914 killed all mice up to a dilution of 10^{-5} and the Victoria strain to a dilution of 10^{-4}. Both viruses titered higher in the presence of normal serum than when previously assayed with buffered saline alone. Brains from final mouse passages were bacteriologically sterile and retained their infectivity after storage in a dry ice box for several months.

Behavior in Eggs.—After repeated mouse passages, it became difficult to induce recognizable infections in embryonated eggs with the viruses. Thus, the California strain 11914, after 11 passages in suckling mice, and the Victoria strain, after 14 mouse passages, no longer infected chicken embryos on inoculation by the allantoic route, as judged by the usual criteria of hemagglutinin formation and death. After 22 mouse passages, however, infectivity

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for chicken embryos was demonstrated for the Victoria strain by using amniotic inoculation in 7- to 9-day-old eggs. This strain was then reidentified by means of an antihemagglutination test. In regard to hemolysis, hemagglutination, and the ability to sensitize chicken erythrocytes to agglutination by serums from patients recovering from infectious mononucleosis,[5] the Victoria strain appeared to have been unmodified by the mouse brain passages. By contrast, repeated attempts to cultivate the California strain 11914 in embryonated eggs after the eleventh mouse passage were indecisive. This strain of virus could be carried through alternate egg-suckling mouse brain passages but, on continuous egg passage, the infectivity of egg fluids disappeared. On intracerebral inoculation of 12, 14-day-old chickens, the California strain, after 22 passages in suckling mice, still produced neurological manifestations typical of ND. This was demonstrated by inoculating the chickens with heat-inactivated serum mixed with virus. In doses of 10^{-2}, 5 of 6 chickens died within three to four days when normal chicken serum was employed, whereas none of the chickens protected with NDV-immune serum succumbed.

PATHOLOGY

A total of 22 NDV-infected mice were examined, 12 infected with the Victoria strain and 10 with the California strain 11914. Twenty-four control mice, as well as 1 monkey, were examined.

Technique.—Mouse tissues were fixed in 10 per cent neutral formalin saline, and paraffin sections were stained by hematoxylin-eosin and the trichrome method. Gross lesions were not seen in the brain after slicing the fixed tissues.

Histology.—In the brains of the mice infected with the Victoria strain, there was generalized capillary congestion easily detected, even under low magnifications. More characteristic, however, there were focal areas of loosening of the ground substance with mobilization of microglia in adjacent parts, all progressing to a spongy state. Coagulation necrosis and shrinkage of nerve cells were noted particularly in the gyrus hippocampi, in the temporal lobes, and to a lesser extent in the thalamus. A minimal degree of perivascular infiltrations by round cells was occasionally seen. There were no hemorrhages, meningeal infiltrations, or lesions in the spinal cord. In mice injected with the California strain 11914, the same type of changes was observed but focal lesions were less numerous and there was not the same degree of severity of alteration.

Control mice (suckling and adult) were injected with diluent alone and with diluent

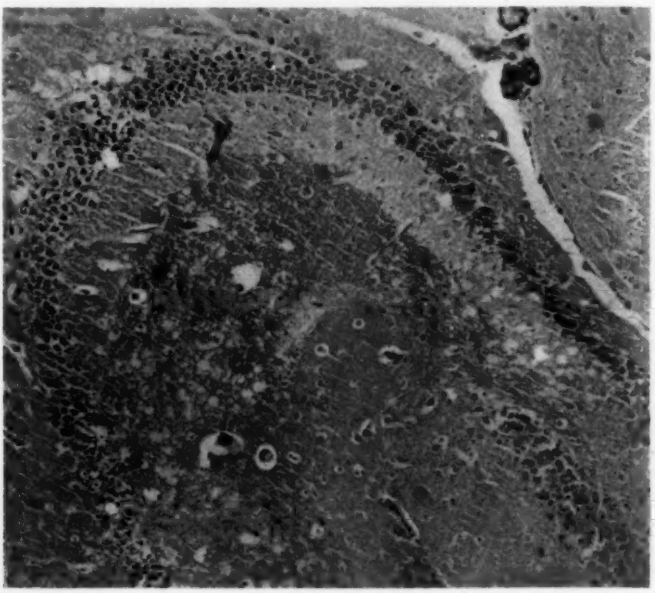

Fig. 1—Mouse, 4 weeks old, sixteenth passage, fourth day, strip necrosis of the gyrus hippocampi and vacuolization of ground substance. Victoria strain of Newcastle disease virus. Hematoxylin-eosin stain. x 100.

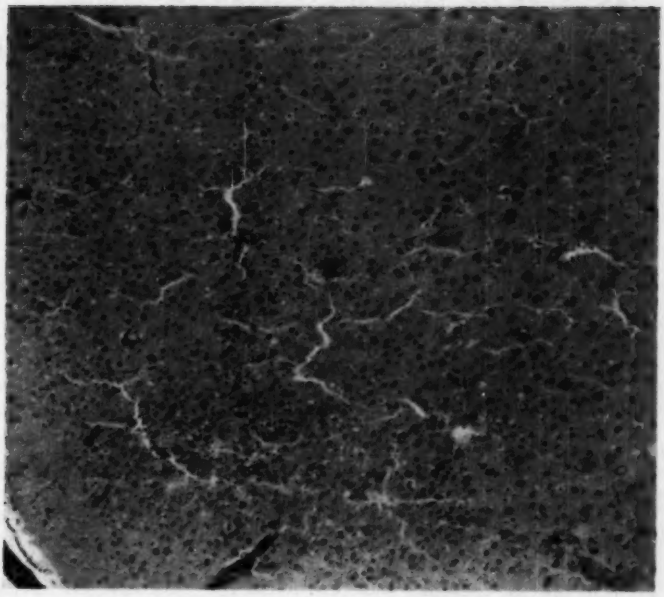

Fig. 2—Mouse, 15 days old, fifth passage, eighth day, an area of microglial mobilization in the cortex of the temporal lobe. California strain 11914 of Newcastle disease virus. Hematoxylin-eosin stain. x 100.

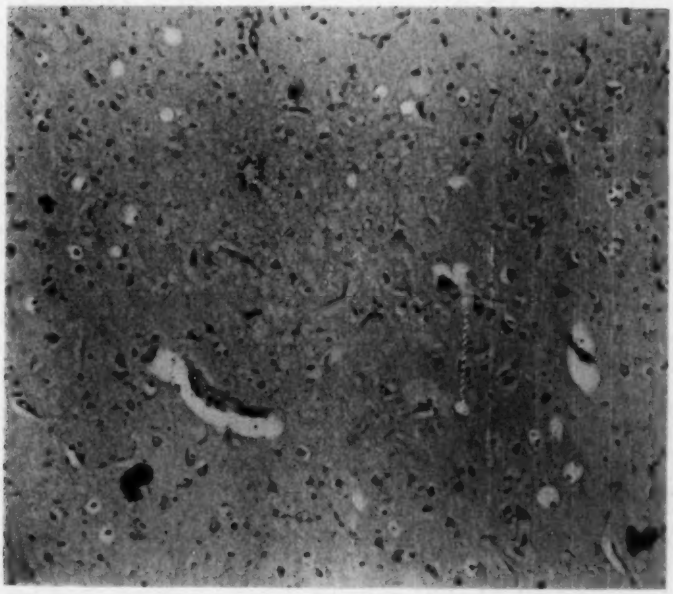

Fig. 3—Monkey, first passage, seventh day, cerebral cortex, a small area of vacuolization of ground substance, early mobilization of microglia and slight perivascular infiltration. Victoria strain of Newcastle disease virus. Hematoxylin-eosin stain. x 100.

and sterile normal brain and sacrificed at intervals of three, six, and nine days. In most animals, lesions were not produced. In a few, however, there were small areas of partial destruction of white matter in the corpus callosum and fornix, in which there were phagocytes containing blood pigments and lipoid debris. All of these were assumed to be due to the trauma of inoculation.

In the 1 monkey inoculated with the Victoria strain, the brain and spinal cord were examined in the routine way. A single small area of loosening and edema of ground substance was present in the parietal cortex. Sections of the midbrain at the level of the third nerve roots showed no lesions in the oculomotor nuclei, red nuclei, and substantia nigra. In the gyrus hippocampi in an area corresponding to Sommer's sector, there was a marked loss of nerve cells. In the adjacent tissue, there were scattered petechial hemorrhages and moderate round cell infiltration about nearby vessels. There was slight patchy round cell infiltration of the meninges and choroid plexus. Lesions were not noted in the spinal cord.

DISCUSSION

Two strains of NDV, which behaved alike in suckling mice, exhibited differences of behavior on transmission to adult mice by intracerebral inoculation. While both strains produced clinical illness and pathological lesions, only the brains of adult mice inoculated with the Victoria strain were infective for other mice. With an incubation period of five to eight days, it would appear unlikely that the pathological lesions produced by the California strain 11914 were due to simple toxicity. An alternative hypothesis is that this virus proliferated in adult mouse brain, as indicated by production of the pathological lesions described above, but failed to reach a fully infective stage of its reproductive cycle. This failure may have been due to some inadequacy of the host cells. Schlesinger[4] described a somewhat analogous situation for influenza virus. He reported that certain non-neurotropic strains of this virus proliferated sufficiently in mouse brain to produce demonstrable increases in hemagglutinating and complement-fixing antigens, although a concomitant decrease of infectivity took place. Another suggestion would be that the California strain 11914 of NDV, although proliferating in adult mouse brain, was neutralized in the process of removal of infected tissue. This hypothesis seems unlikely if one considers that the Victoria strain, which is antigenically closely related, was readily recovered from mouse brains under identical circumstances.

An additional peculiarity of the California strain was that its pathogenicity for embryonated eggs was altered following continued passage in suckling mice. Possibly, this resulted from a closer adaptation to brain tissue, for it was still fully virulent for chickens on intracerebral inoculation. Whether these mouse-adapted viruses might have value as vaccinating agents against ND was not assayed, due to lack of suitable facilities for handling young chicks.

SUMMARY

1) The Victoria and the California 11914 strains of Newcastle disease virus, after passage in suckling mice, lead to characteristic clinical and pathological findings on intracerebral inoculation of adult mice.

2) The Victoria strain was carried through ten successive passages in the brains of adult mice without change in the fundamental properties.

3) Continued passage of the California strain 11914 was possible only in suckling mice. The brains of adult mice which had developed a characteristic illness following inoculation with this virus were not infective for other mice. In the course of repeated mouse passages, the California strain could not be cultivated in eggs by any of the usual methods which were tried.

4) Newcastle disease virus produces a definite encephalitis, in both suckling and adult mice, of a progressive type usually fatal within seven to ten days. In this experiment, its lesions appeared concentrated in the gyrus hippocampi and the frontal and temporal lobes. The brain was the only organ in which lesions were observed.

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The Effects of Several Antibiotic Products in Feed on Experimental Ulcerative Enteritis in Quail

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THE EARLIER reports by us on the benefits provided by streptomycin against experimental ulcerative enteritis in bobwhite quail (*Colinus virginianus*) were the first record of successful treatment of this disease by any antimicrobial agent.[1, 2] The possible usefulness of information on other antibiotic preparations and on the feasibility of applying them in the feed prompted the studies described in this report. These included an evaluation of a preparation that is available early in the manufacturing stages of streptomycin, of a product that is a residue in the manufacture of aureomycin, and of chloromycetin.

MATERIALS AND METHODS

The antibiotic preparations studied were: MK-42, containing 30 Gm. of streptomycin "Base" per pound; aurofac, containing 1.8 Gm. of aureomycin per pound; and pure chloromycetin.

The antibiotic preparations were provided the quail in mash. The MK-42 was used in the ratio of 1 lb. to 100 lb. of feed and in four lower amounts, giving approximate concentrations of streptomycin base of 300, 150, 60, 30, and 15 mg. per pound of feed. Aurofac was used only in the ratio of 0.5 lb. per 100 lb. of feed, providing 9 mg. of aureomycin per pound of feed. Chloromycetin was incorporated in the feed at levels at 1,000, 500, and 250 mg. per pound. With two exceptions, administration of the antibiotics was begun seven days before the quail were exposed to the infective material of ulcerative enteritis. The exceptions were the initial study of MK-42 (group 3, table 1) and the one trial of aureomycin where, in the course of other studies, the use of the antibiotics was started when the quail were hatched. In all cases, the antibiotics were givn continuously until thirty days postexposure to the disease.

Twelve groups of 18 to 29 quail were utilized in the study. Two of the groups did not receive antibiotics; these served also as control groups for testing concurrently various regimes of streptomycin in drinking water.[2] The procedures used in reproducing and identifying the disease and in handling the birds have been described.[1, 2]

Eighty-nine quail from eight of the treated groups having 18 or more survivors were reëxposed to the infectious material thirty-five days following the initial exposure. The other portions of the groups were segregated and held for observation.

RESULTS

A suppressive effect on the development of ulcerative enteritis was demonstrated for MK-42, the streptomycin product, and for the purified chloromycetin, whereas, under the conditions imposed, aureomycin provided no benefit. The mortalities for the various groups, as recorded in table 1, showed that the groups of quail that received 30 mg. or more of streptomycin base per pound of feed (groups 3 to 7, inclusive) or 500 or 1,000 mg. of chloromycetin per pound of feed were provided a high degree of protection against the disease. Lower levels of these antibiotics gave less adequate protection: mortalities of 43 per cent and 17 per cent occurred in the groups that received 15 mg. of streptomycin and 250 mg. of chloromycetin, respectively, per pound of feed as compared to the maximum of 4 per cent that occurred in any other group treated with these materials. The group that received the one level of aureomycin that was tested (9 mg. per pound of feed) had a mortality of 23 out of 26 quail, which was essentially the same as that of the nontreated group.

The deterrent effect on the disease, or the lack of it, from the lowest dosages of streptomycin and chloromycetin and from aureomycin was evidenced by the patterns

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Grateful acknowledgment is made to the Indiana Department of Conservation, supplier of the quail eggs and pens, to Merck and Company, Inc., Rahway, N. J., Lederle Laboratories, Inc., Pearl River, N. Y., and Parke, Davis and Company, Detroit, Mich., suppliers of streptomycin, aureomycin, and chloromycetin products, respectively, and to Dr. D. E. Stulken and Mr. R. A. Hoffman, who assisted in important management problems involved in handling the quail.

in which deaths occurred as well as by mortalities. Deaths from ulcerative enteritis were rather evenly distributed between two and twenty-five days postexposure in the group that received feed containing 15 mg. of streptomycin per pound and between four and sixteen days postexposure in the group having feed with 250 mg. of chloromycetin per pound. The deaths in the group which received aureomycin occurred between two and twelve days postexposure, with an average survival period of 5.7 days; this death pattern was comparable to that of the control group.

TABLE I—The Effects of Several Antibiotics on Ulcerative Enteritis in Quail when Given Continuously in the Feed from Before Exposure to Thirty Days Postexposure

Group No.	Compound and concentration in the feed		Milligrams of antibiotic in each pound of feed	Ratio of number of quail that died to the number exposed
1*	None		None	23/24
2				20/23
3*	MK-42**	1.00%	300	0/18
4	MK-42	1.00%	300	0/23
5	MK-42	0.50%	150	0/24
6	MK-42	0.20%	60	0/22
7	MK-42	0.10%	30	1/24
8	MK-42	0.05%	15	9/21
9	Aurofac†	0.50%	9	23/26
10			1,000	0/25
11	Pure		500	0/25
12	chloromycetin		250	5/29

*These two tests were conducted at one time, all others at another. **Active principle: streptomycin 'base'. †Active principle: aureomycin.

The reëxposure of 89 of the survivors of eight of the treated groups resulted in deaths of 77 (86.5%) of the birds. The survivors were distributed among all of the groups. Among 95 birds of the same origin that were not reëxposed but held only to detect the possible occurrence of late deaths from the initial exposure, 2 died from enteritis during a thirty-day observation period.

DISCUSSION

The benefits derived from the use in quail rations of MK-42, a relatively impure preparation of streptomycin, and of chloromycetin in preventing experimental ulcerative enteritis warrants their application in attempts to suppress the naturally occurring disease. Approximate minimal dosages that could suppress the type of experimental exposure and disease employed appears to have been defined for these two antibiotics. The minimal dosage for streptomycin base was 30 mg. per pound of feed and that for chloromycetin was 500 mg. per pound.

A comparison between the apparent minimal effective dosage of streptomycin as provided in the MK-42 product in the ration and the minimal concentration (1 Gm. per gallon) of pure base tested and found effective in drinking water[2] indicates that nearly four times more of the base needs to be consumed in the mash than in the water. This figure is based on the observation that quail 8 to 10 weeks old consume approximately 20 Gm. of mash and 15 ml. of water daily. A confirmatory evaluation of the suppressive effect of 1 Gm. of streptomycin base per gallon of water, as previously described,[2] was conducted at the same time, utilizing the same infectious inoculum, as the trial on the graded dosages of MK-42.

The previous observations[1, 2] that a majority of quail that survived in groups successfully treated with streptomycin remained highy susceptible to the disease were confirmed in this study, and the principle was found to apply also to chloromycetin-treated birds. The capacity of quail to develop immunity to the experimentally produced disease was demonstrated in survivors of naturally occurring outbreaks.[1]

SUMMARY

An evaluation was made of the effect of streptomycin "base" in an experimental product, MK-42, or aureomycin in aurofac, and of pure chloromycetin on experimental ulcerative enteritis.

The provision of the MK-42 product at various levels in the ration prior to exposure to the disease and for thirty days thereafter indicated that a 0.1 per cent concentration, giving 30 mg. of base per pound, was the approximate minimal effective dosage.

Similar provision of feed containing chloromycetin showed 500 but not 250 mg. per pound to be highly effective.

A concentration of 0.5 per cent of aurofac in the feed, providing 9 mg. of aureomycin per pound, was without any deterrent effect on the disease.

The birds successfully treated remained

without any appreciable resistance to subsequent experimental exposure to the disease.

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Streptomycin Studies in Ulcerative Enteritis in Bobwhite Quail. II. Concentrations of Streptomycin in Drinking Water Suppressing the Experimental Disease

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PREVIOUSLY[1] WE reported therapeutic properties for streptomycin in ulcerative enteritis in bobwhite quail (*Colinus virginianus*). The antibiotic was administered in the drinking water at a level of 15 Gm. per gallon beginning two days prior to exposure of the quail to infective material, on the day of exposure, or two or four days thereafter, and continued for five or ten days. Further studies, as given in this report, sought to determine the validity of our earlier observations and to explore the possibilities of more economical usage of the antibiotic.

MATERIALS AND METHODS

Two trials were conducted in series, one (trial 1) beginning in late summer and the other (trial 2) in midautumn. In both trials, 20 groups of 20 to 25 quail and one of 10 quail, all 8 to 9 weeks old, were used in evaluating fifteen regimes of treatment.

Two forms of the antibiotic were used, namely, the purer "base," as a calcium chloride complex, and the "streptomycin mixture," containing 0.351 Gm. of base per gram. In one or the other form, streptomycin was given in the drinking water at seven dosage levels, varying in terms of concentration of antibiotic base from 1 to 15 Gm. per gallon of water. Administration of the antibiotic was begun before exposure of the quail to the disease factor and continued for eight or thirty days thereafter in three groups, on the day of exposure and continued for three or five days in six groups, and at the end of the second postexposure day and continued for three or five days in ten groups.

The infectious material used to produce the disease was prepared from triturated intestines of quail that had died of typical ulcerative enteritis. Just prior to any exposure procedure, an appropriate amount of frozen, triturated intestines was thawed, diluted to a final concentration of one part to 25 of physiological saline, and filtered through cheesecloth. A 1-ml. quantity of the filtrate was introduced into the crop of each bird. Portions of a single preparation were used for all inoculations excepting for the reëxposure of the birds in trial 2.

Approximately, one half of the quail that survived in five of the treated groups in each trial were segregated and exposed again thirty-five (trial 1) or thirty-seven (trial 2) days after the initial exposure to determine their immunity status to the disease. The other half of each group was held for further observation. Only in trial 1 were suitable controls available for this phase of the study.

The facilities for handling the quail and the criteria for recognition of the disease were the same as those previously described.[1] *

RESULTS

A summary of the mortality and its pattern in the groups of quail that were treated and those not treated with streptomycin is shown in table 1. In trial 1, the experimental exposure to ulcerative enteritis caused death of 23 of 24 birds that received no treatment; all of the deaths occurred between two and fourteen days postexposure. Within this same period, 18 (6.6%) of 272 birds died in the 15 treated groups, each of which was subjected to a different regime of antibiotic. By comparable consideration of the results in trial 2, 20 (88%) of 23 quail died from the uninhibited disease, and 32 (21%) of 152 birds died in the seven treated groups. All of the six concentrations of the antibiotic, varying from 1.75 to 15 Gm. of base per gallon, used in short-term treatments, and the concentration of 1 Gm. per gallon, used in long-term treatment, exerted a marked suppressive effect on the disease.

The time when treatment was started in relation to the day of exposure and possibly

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This investigation was done in coöperation with the Indiana Department of Conservation, which supplied quail eggs and pens, and with Merck and Company, Inc., Rahway, N. J., supplier of the streptomycin.

*The assistance of Dr. D. E. Stullken and Mr. R. A. Hoffman in the details of management is gratefully acknowledged.

the duration of the treatment were of greater importance than the concentration of the antibiotic in determining the pattern

TABLE 1—The Suppression of Experimental Ulcerative Enteritis in Quail by Streptomycin in Drinking Water

	Group no.	Grams of streptomycin base per gallon	Deaths in relation to exposure time, days. (Shaded areas show treatment periods.)	Number that died/ number exposed
Trial 1	1	0	1 2 1 7 3 3 2 2 1 1 1	23/24
	2	15	1/20	
	3		0/25	
	4	10	5/25	
	5		1/24	
	6		2 2 3	7/24
	7		1 1 1 1 1 0	13/24
	8	5.24*	0/24	
	9	5	0/24	
	10		8	8/24
	11		3 1 4	8/24
	12		3 1 3	8/24
	13	.00	0/10	
Trial 2	14	0	1 5 3 1 1 2 3 3 1	20/23
	15	15	1 1 1 3 6	13/24
	16	10	2 1	3/22
	17	3.26*	2 3 1	7/22
	18	5	1 1	2/20
	19	3.5*	1 1 2 1	4/21
	20	1.75*	5 2 2 1	10/23
	21	.00	0/20 1	

*The antibiotic was provided in these cases in 15, 10, or 5 Gm. per gallon of "streptomycin mixture." **The antibiotic was provided in these cases as 1 Gm. per gallon of streptomycin "base" given continuously from hatching day.

and extent of losses by death. Thus, in trial 1, irrespective of the dosage of antibiotic, no deaths occurred during the period of treatment in the eight groups of birds to which the antibiotic was provided before or on the day of exposure. Furthermore, in the seven groups that received short-term treatment beginning before or on the day of exposure, deaths did not occur during the periods between the days when the treatment was withdrawn and the fourteenth postexposure day when all deaths had occurred in the nontreated group. Of the 18 quail that died in the treated groups in this trial, all were in four groups to which the antibiotic was introduced at the end of the second postexposure day. Six of these occurred prior to the addition of the antibiotic to the drinking water, seven during the first two days of treatment, and five during the nine-day period between the end of treatment in two groups and the time required for deaths among the controls. Groups 7 and 12, in which the five late deaths occurred, had received the antibiotic for only three days.

The benefits revealed from the antibiotic by the seven treated groups in trial 2 paralleled generally those of trial 1 and indicated therapeutic value for two lower concentrations of streptomycin, 1.75 and 3.5 Gm. per gallon, in short-term treatment. Death of 26 (81%) of 32 birds that died during the first two weeks postexposure (which period again accounted for the deaths in the control group) occurred in the six groups which received treatment beginning at the end of the second postexposure day and ending five days later. Fourteen of the 26 quail died before treatment was given and 12 during the first two days of treatment. The other six deaths came in three groups of birds four to seven days after the withdrawal of the antibiotic. Of these three groups, group 15 had received the highest concentration of the antibiotic and groups 19 and 20 the two lower dosages.

From the fourteenth day postexposure, when all deaths in the control groups had stopped, to the end of the thirty-day observation period, 40 quail died in ten of 17 groups that had received short-term treatments. A direct correlation did not exist between the occurrence and number of deaths at this time and the regimes of treatment.

The reëxposure of about one half of the survivors of five treated groups in each trial thirty-five or thirty-seven days after the initial exposure caused deaths of 82.8 per cent of 101 birds. As shown further in table 2, the average number of days until death of the birds in the various groups revealed a high susceptibility of the majority of the birds to the disease. The mortality pattern in each of the groups indicated the absence of any marked differences in the development of immunity by variations in dosages of the antibiotic, the time when the treatment was applied, or its duration.

TABLE 2—Reëxposure of Quail Exposed to Ulcerative Enteritis About Five Weeks Previously and Treated with Streptomycin

Trial No.*	Group No.*	No. that died/ No. exposed	Days to death postexposure
1	Controls	9/10	5.8
	2	8/10	4.6
	3	11/12	4.6
	5	9/12	4.3
	8	10/12	6.7
	9	12/12	7.0
2	16	6/9	5.8
	17	5/8	3.8
	18	6/7	5.8
	19	5/9	4.8
	21	10/10	5.2

*The trial and group numbers are the same as those used in table 1.

DISCUSSION

The results provided strong confirmation of our earlier observation that streptomycin exerted a suppressive effect on the development of experimental ulcerative enteritis[1] and showed that much smaller amounts of antibiotic than previously used are of comparable value.

The evaluation of 15 regimes of treatment, involving seven concentrations of antibiotic varying from 1 to 15 Gm. per gallon of drinking water and four treatment periods beginning before, on, or after exposure, showed all to be highly effective. No adverse effects of the antibiotic itself were detected, irrespective of whether it was given at a level of 15 Gm. per gallon for ten days to quail about 8 weeks of age or in a concentration of 1 Gm. per gallon for twelve weeks beginning when the birds were 1 day old. A unit of base appeared to be equally effective, as represented either by the calcium chloride complex or by the streptomycin mixture. Trial 1 was conducted during the summer, when the natural disease is most often observed, and the results appeared to be equal to those obtained in trial 2 in the fall of the year.

The occurrence of post-treatment deaths from enteritis, which amounted to 25 to 45 per cent of the birds in some groups, is subject to several interpretations. In several groups, particularly two which were given the antibiotic for only three days, beginning two days after exposure, it appears likely that the deaths that occurred within the first two weeks after exposure may have been due to inadequate treatment. This reasoning seems less plausible when applied to the deaths that occurred later, beyond fourteen days postexposure, when mortality in the control groups had stopped. These late deaths in the treated groups may have resulted from simple spread of the disease in some groups. Also, spread of the disease between groups seems a very likely possibility. For example, group 4 had five deaths between fourteen and thirty days postexposure, whereas deaths did not occur during the same time in groups 9 and 3, which had received lower or higher levels of antibiotic than group 4. A better understanding of the means whereby the disease is disseminated will undoubtedly depend in large measure on clarification of the questionable etiology of the disease.[2, 3, 4]

In any practical applications of these results, it should be recognized that successful treatment of any birds would likely leave many of the survivors without appreciable resistance to reëxposure. Hence, either periodic or longer terms of treatment or the improvement of sanitary precautions would appear to be in order when a major threat by the disease exists.

SUMMARY

Fifteen regimes of treatment were studied, involving seven concentrations of streptomycin varying from 1 to 15 Gm. per gallon of drinking water and four treatment periods beginning before, on, or after exposure to ulcerative enteritis. Among 19 treated groups containing 424 quail, a mortality of 21 per cent occurred; 88.7 per cent of these deaths occurred prior to or early in the course of treatment or at a considerable time thereafter. In two groups of nontreated quail, the disease caused death of 91 per cent of 47 birds.

A concentration of 1.75 Gm. of antibiotic "base" per gallon given two days postexposure and continued for five days, the minimum regime used, was beneficial in suppressing the expectable course of the disease.

The continuous administration of water containing 1 Gm. of the base per gallon from hatching time until 12 weeks of age was without adverse effects in itself and totally suppressed the lethal or other observable effects of exposure of quail to infective material at 8 weeks of age.

Suppression of the disease by the antibiotic as late as two days postexposure, when deaths of quail had already started, left the majority of the birds with little, if any, resistance to the disease.

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Studies in Avian Leukosis. VI. Serial Passage of a Highly Malignant Strain of Visceral Lymphomatosis in Young Chickens

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THE DEVELOPMENT of malignant strains of visceral lymphomatosis from naturally occurring cases by their serial passage in chickens has been reported by Olson[5, 6] and Burmester and Prickett.[1] Davis and Doyle[2, 3] and Davis, Andrews, and Doyle[4] have also reported results of serial passage of avian visceral lymphomatosis in chickens.

MATERIALS AND METHODS

Between October, 1947, and May, 1949, two series of chicken passages of a highly malignant strain of visceral lymphomatosis were carried out. The purpose of these serial passages was to maintain in chickens a strain of visceral lymphomatosis of known and heightened virulence in order to furnish a continuous, dependable, and uniform source of material for the inoculation of chicken embryos in studies of avian leukosis by the use of chicken embryo techniques which were being undertaken during this period. The strain used, furnished for this purpose by the U. S. Regional Poultry Research Laboratory at East Lansing, Mich., was their strain RPL 16, the development of which was reported by Burmester and Prickett[1] in 1945. This strain not only produces a very high percentage of visceral tumors but also has a marked affinity for muscle, and produces a high percentage of large tumors in the pectoral muscle following inoculation in that area. The pectoral muscle tumors are a particularly useful source of material for inoculums in chicken embryo studies.

In October, 1947, frozen material consisting of a tumor of the pectoral muscle of a chicken infected with visceral lymphomatosis of RPL strain 16 was received from Dr. G. E. Cottral of the U. S. Regional Poultry Research Laboratory. This material was thawed at room temperature and triturated in sterile physiological saline solution in a concentration of approximately 1 : 10. Twenty White Rock chickens, 27 days of age, were each inoculated with 2 cc. of the suspension into the pectoral muscle. Twelve (60%) of these chickens developed visceral lymphomatosis, the first death occurring ten days after inoculation. All cases of visceral lymphomatosis showed large tumors of the pectoral muscle and greatly enlarged livers, and many also showed enlarged spleens, kidneys, or hearts. Similar gross lesions were found consistently in later serial passages, except that in a few cases breast tumors did not occur, and in many cases pronounced tumors of the pancreas, mesentery, gizzard, and proventriculus walls also occurred with considerable frequency.

Between October, 1947, and July, 1948, 30 serial passages were carried out successfully. In all but passage 8, where breast tumor only was used, the inoculum was prepared from breast tumor and liver in an approximately 10 per cent suspension. The amount of inoculum injected into each chicken was 1.5 cc. in passages 2, 3, and 4, and 2 cc. in all later passages. Chickens from each passage were killed when moribund, and breast tumors and organs were collected aseptically and stored in a deep freeze unit at —20 C. for later use as inoculums. Results of this series of passages are summarized in table 1. Tissues were saved for histological study from all chickens from which tissues were frozen and from all chickens from which fresh tumor material was used as inoculums either in later serial passages or in chicken embryo experiments (from 1 to 11 chickens in each passage, ave. 3.7 for all passages). All chickens examined in this way proved to be undoubted cases of visceral lymphomatosis. Of the organs studied histologically in the various passages, from 60 to 100 per cent (ave. 98.25% for all 30 passages) of the livers, from 40 to 100 per cent (ave. 97.52%) of the spleens, from 62.5 to 100 per cent (ave. 94.96%) of the kidneys, and from 0 to 100 per cent (ave. 77.35%) of the hearts were tumorous.

In November, 1948, a second series of passages of RPL strain 16 of visceral lymphomatosis was begun. Frozen tumor material from the same source as mentioned previously was used again to initiate this series of passages. Fifteen passages were carried out between November, 1948, and May, 1949. Two passages in the series were made from passage

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11 (these are designated as 12 and 12A in table 2), the first using fresh breast tumor and liver as inoculum, and the second using breast tumor which had been held frozen at —20 C. in the deep freeze unit for twelve days. It was the second of these from which the donor for passage 13 was selected. With this exception, procedures similar to those used in the preceding series of passages were followed, and similar results were obtained. Inoculums were prepared from breast tumor and liver in an approximately 10 per cent suspension in all passages excepting 1, 2, and 12A, where breast tumor alone was used. Two cubic centimeters of the inoculum were injected into each chicken in all passages excepting 1, where 1.5 cc. was used, and in passage 13, where 1 cc. was used. Results of this series of 15 passages are summarized in table 2, which includes percentages of gross lesions at autopsy in the organs most commonly involved. In other organs frequently involved, from 0 to 68.6 per cent of the birds in the various passages (ave. 29.87% for the 15 passages) grossly showed tumorous gonads at autopsy, from 0 to 26.1 per cent (ave. 12.03%) showed tumorous proventriculus walls, from 0 to 23.8 per cent (ave. 7.94%) showed tumorous pancreases, from 0 to 19.1 per cent (ave. 6.30%) showed tumorous gizzards, from 0 to 19.1 per cent (ave. 6.02%) showed tumorous hearts, from 0 to 18.2 per cent (ave. 4.49%) showed tumorous mesenteries, and from 0 to 9.5 per cent (ave. 1.50%) showed tumorous adrenals. Tissues were saved for histological study from all chickens used as donors for either later serial passages or for chicken embryo experiments, or from which tissues were frozen and stored in the deep freeze unit for later use as inoculums (from 1 to 4 chickens in each passage, ave. 2.7 for all passages). All chickens whose tissues were examined microscopically proved to be marked cases of visceral lymphomatosis. Of the organs studied histologically, 100 per cent in all passages of the livers, spleens, and gonads, from 50 to 100 per cent (ave. 96.42%) of the kidneys, and from 0 to 100 per cent (ave. 98.28%) of the spleens were tumorous.

TABLE 1—Thirty Serial Passages in Young Chickens of Avian Visceral Lymphomatosis of RPL Strain 16 of Series D1

Serial passage	No. in group	Age (days)	Visceral lymphomatosis (%)	Ave. days after inoculation before death of cases	Tumorous breasts (%)	Tumorous liver (%)
1	20	27	60.0	19.66	83.3	91.6
2	24	37	100.0	10.17	96.0	100.0
3	24	17	91.7	6.73	95.7	95.7
4	15	51	100.0	7.93	60.0	93.3
5	10	57	80.0	13.75	87.5	100.0
6	13	14	100.0	7.60	92.3	100.0
7	18	22	100.0	9.94	94.4	100.0
8	21	30	100.0	10.38	100.0	100.0
9	14	30	100.0	11.93	100.0	100.0
10	21	34	57.1	30.41	83.3	100.0
11	24	41	87.5	25.52	95.2	95.2
12	20	42	85.0	9.82	94.1	100.0
13	20	45	85.0	8.88	100.0	100.0
14	20	42	100.0	8.90	80.0	95.0
15	20	49	100.0	9.40	100.0	95.0
16	20	49	100.0	7.85	50.0	100.0
17	20	50	100.0	8.00	80.0	100.0
18	20	50	100.0	7.85	100.0	100.0
19	20	9	100.0	5.95	95.0	100.0
20	7	20	100.0	6.43	100.0	100.0
21	13	14	100.0	6.85	76.9	92.3
22	20	18	100.0	7.75	95.0	100.0
23	20	25	100.0	6.60	85.0	90.0
24	20	32	95.0	13.21	100.0	78.9
25	20	23	90.0	10.33	45.7	80.0
26	20	35	100.0	7.75	85.0	95.0
27	20	36	85.0	8.71	100.0	94.1
28	19	38	84.2	10.19	87.5	93.8
29	21	19	100.0	9.05	100.0	95.2
30	20	50	95.0	10.68	100.0	89.6
Ave.		33.5	90.18	10.61	88.73	95.82

DISCUSSION

It is interesting that results of serial passages of visceral lymphomatosis of strain RPL 16 of both series described here are closely comparable to the results of the later passages in the development of this strain, as described by Burmester and Prickett.[1] The high percentage of death loss and rapidity of death after inoculation was, in fact, greater in both of these series than in theirs. This is true, even if only passages in their series after the high malignancy was established are considered, and even though younger chickens were generally used in their passages than in ours. Furthermore, strain RPL 16 developed the most consistently high malignancy

TABLE 2—Fifteen Serial Passages in Young Chickens of Avian Visceral Lymphomatosis of RPL Strain 16 of Series D2

Serial passage	No. in group	Age (days)	Visceral lymphomatosis (%)	Ave. days after inoculation before death of cases	Tumorous breasts (%)	Tumorous livers (%)	Tumorous spleens (%)	Tumorous kidneys (%)
1	20	38	35.0	18.57	100.0	85.7	85.7	57.1
2	22	65	81.8	10.11	100.0	94.4	88.9	94.4
3	17	39	88.2	8.53	100.0	87.5	81.2	93.8
4	20	46	90.0	8.53	100.0	82.3	76.4	76.6
5	19	53	84.2	11.00	100.0	100.0	100.0	93.8
6	20	45	100.0	7.90	100.0	100.0	100.0	100.0
7	23	52	91.3	7.81	100.0	90.5	100.0	90.5
8	25	40	88.0	9.13	97.2	83.3	97.2	97.2
9	12	37	91.6	10.82	100.0	90.9	81.8	100.0
10	17	33	100.0	8.00	94.1	94.1	94.1	100.0
11	18	26	100.0	9.88	100.0	94.4	94.4	94.4
12	21	28	100.0	7.42	100.0	90.5	90.5	95.2
12A	20	47	70.0	14.64	100.0	86.6	73.3	80.0
13	25	39	92.0	9.70	100.0	100.0	86.9	100.0
14	25	49	92.0	9.70	100.0	91.4	95.7	95.7
15	13	47	100.0	9.30	100.0	100.0	100.0	100.0
Ave.		41.5	87.75	10.06	99.45	91.97	90.38	91.41

of any of the four strains developed by Burmester and Prickett.[1]

The exceptionally high and rapid death loss in these two series of passages of strain RPL 16 is particularly striking as compared with results of inoculation with similar lymphomatous material from field cases. For example, in two such transmission experiments carried out at this laboratory, the death loss in one was 38.8 per cent of inoculated birds in an average of 174 days after inoculation,[2] and in the other, 43 per cent of inoculated birds in an average of 171.5 days after inoculation.[3] This can be compared with average death losses of 90.18 and 87.75 per cent in averages of 10.81 and 10.06 days after inoculation in the two series of passages described here.

SUMMARY

Thirty serial passages in chickens of avian visceral lymphomatosis of RPL strain 16 were carried out successfully between October, 1947, and July, 1948. The incidence of the disease varied from 60 per cent in the first passage initiated with frozen material to 100 per cent in many later passages (ave. 90.18% for the 30 passages). Average time from inoculation until death of cases varied between 5.95 and 30.41 days (ave. period of 10.61 days for the 30 passages). Between 50 and 100 per cent of the cases in the various passages showed breast tumors at the site of inoculation at autopsy (88.73% of the cases in all 30 passages). Between 78.9 and 100 per cent showed tumorous livers (ave. 95.85% in all passages).

A second series of 15 passages of RPL strain 16 of visceral lymphomatosis in chickens was carried out successfully between November, 1948, and May, 1949. The incidence varied from 35 per cent in passage 1 and 70 per cent in passage 12A, where frozen tumor materials were used as inoculums, to 100 per cent in several other passages (ave. of 87.75% for the 15 passages of the series). The average period between inoculation and death of cases varied between 7.42 and 18.57 days (ave. of 10.06 days for all passages). Between 94.1 and 100 per cent of the cases in the various passages (ave. 99.45%) showed breast tumors at the site of inoculation at autopsy, and between 82.3 and 100 per cent (ave. 91.97%) showed tumorous livers. Tumorous spleens and kidneys also occurred with high frequency, while the gonads, heart, pancreas, proventriculus, gizzard, mesentery, and adrenals also showed gross lesions of visceral lymphomatosis at autopsy with less frequency.

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Studies on the Use of 2-Amino-5-Nitrothiazole in the Control of Enterohepatitis (Blackhead) in Turkeys

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THE PRELIMINARY work of several investigators using 2-amino-5-nitrothiazole* (ANT) to combat enterohepatitis in turkeys has been summarized by Waletzky.[1]

This experiment was undertaken to determine if more effective and economical ways of administering ANT could be established.

MATERIALS AND METHODS

Test conditions simulating those in the field were devised to ascertain the effect of ANT on the course of natural outbreaks of enterohepatitis.

Four hundred and forty-eight 8-week-old poults were divided equally into eight test groups (designated lots A, B, C, D, E, F, G, and H). The 56 birds in lot A were kept as untreated controls for all test groups. Turkeys in all lots in this experiment were kept under similar conditions of husbandry and exposure to infection.

Enterohepatitis was induced in all test groups by methods described by McGregor.[2] Equal amounts of infective material were given in the drinking water to all groups on the same day and were kept before all birds continuously for twelve days. The experiment required forty-seven days.

The experiment was divided into two parts. The first dealt with the prophylactic value of ANT, using birds in lots, B, C, and D. The second concerned the use of the birds in lots E, F, G, and H to test the therapeutic value of the drug.

ANT was given at various concentrations and at different intervals, as shown in table 1. In all medicated groups, ANT was given in the feed, except in the case of lot H, where a soluble form of the drug was put into fresh drinking water daily.

Prophylactic medication was initiated in lots B, C, and D on the same day as infective material was given. ANT was kept before the birds of these three lots in the amounts shown in table 1.

The curative attempts represented by lots E, F, G, and H were devised to resemble field conditions where a rancher may delay use of an expensive drug for a day or two until the extent of disease in his turkey flock has been ascertained and administration of such a drug is warranted. Table 1 indicates the dosage procedure for these four lots.

ANT in soluble form was used in lot H as a curative rather than as a prophylactic treatment, since this form would more likely be used as a therapeutic agent by turkey producers.

RESULTS

The results of the experiment are summarized in table 1.

DISCUSSION

An abnormally virulent form of enterohepatitis infection developed in all lots of turkeys used in this experiment. Losses due to the disease were much greater than those usually observed in the field.

As shown by table 1, ANT used as a preventive agent in the feed, in concentrations lower than 0.05 per cent, had little or no significant effect in a severe outbreak of enterohepatitis. When ANT was given at 0.05 per cent concentration in the feed during alternate weeks, a degree of protection against the infection was afforded, but the protection was not comparable to that offered by ANT given in the same concentration continuously, as was shown in a previous experiment by McGregor.[2] What effect administering ANT during alternate weeks at 0.05 per cent concentration might have in protecting birds exposed to enterohepatitis under normal field conditions has not been determined.

It was evident that ANT, given at the 0.1 per cent level for seven days only, was not enough to promote recovery of birds or to check a severe enterohepatitis enzoötic noticeably, since 51.78 per cent of the birds in lot E died after treatment began.

In contrast to the procedure used in lot

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*Enheptin (Lederle Laboratories).

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E, the method of administering ANT in lot F produced more favorable results. There was a loss of only 25 per cent after treatment began, and the number of deaths decreased sharply three days after use of the drug commenced.

SUMMARY

Four hundred and forty-eight 8-week-old turkey poults were divided into eight equal groups, seven of which were given 2-amino-5-nitrothiazole (ANT) in various ways to combat enterohepatitis. One group was kept as an untreated control. The drug was used as a prophylactic and as a curative agent against severe enterohepatitis outbreak.

Under the conditions of this experiment, the drug given in the feed as a prophylactic and used for forty-seven days had a limited effect, proportional to the concentration administered.

ANT used as a curative treatment in established enterohepatitis enzoötics showed some activity, especially when given in successive concentrations of 0.2 per cent for three days, 0.1 per cent for seven days, and 0.05 per cent continuously thereafter in the feed.

The drug, used in a soluble form as a curative treatment, did not prove markedly effective in stopping a severe outbreak of enterohepatitis, under the conditions of this experiment.

TABLE 1—Dosage Procedures and Results Obtained by the Use of 2-Amino-5-Nitrothiazole in Control of Enterohepatitis in Turkeys

Lot	No. birds	Compound used	Dosage procedure	Losses of birds from enterohepatitis
A	56	Nil	Nil	55(98.21%)
B	56	2-amino-5-nitrothiazole	0.0125% given continuously in the feed	54(96.42%)
C	56	2-amino-5-nitrothiazole	0.025% given continuously in the feed	38(67.85%)
D	56	2-amino-5-nitrothiazole	0.05% given in the feed during alternate weeks	19(33.92%)
E	56	2-amino-5-nitrothiazole	Used as a treatment 2 days after enterohepatitis was apparent. Dose: 0.1% for 7 days in the feed, followed by 0.05% continuously in the feed.	13(23.21%)* 29(51.78%)** 42(75.0%)***
F	56	2-amino-5-nitrothiazole	Used as a treatment 3 days after enterohepatitis was apparent. Dose: 0.2% for 3 days, followed by 0.1% for 7 days, followed by 0.05% continuously in the feed.	13(23.21%)* 14(25.00%)** 27(48.21%)***
G	56	2-amino-5-nitrothiazole	Used as a treatment 3 days after enterohepatitis was apparent. Dose: 0.1% in the feed for 7 days, plain feed for 7 days, followed by 0.1% in the feed for 7 days.	10(17.85%)* 16(28.57%)** 26(46.42%)***
H	56	2-amino-5-nitrothiazole (soluble)	Used as a treatment 2 days after enterohepatitis was apparent. Dose: 1 oz. per 12 gal. water for 14 days, followed by 1 oz. to 24 gal. of water continuously.	3(5.35%)* 26(46.42%)** 29(51.78%)***

*Died from enterohepatitis before treatment began. **Died from enterohepatitis after treatment began. ***Total deaths from enterohepatitis.

The treatment used in lot G was only apparently successful in controlling the severe enterohepatitis outbreak. The 28.57 per cent loss sustained after treatment began was deceiving, since mortality was not quickly reduced by administration of the drug, and deaths occurred at a constant rate until near the end of the experiment.

In lot H, where soluble ANT was given in the drinking water, results were not promising, since 46.42 per cent of the birds died from the disease after treatment was started. Deaths continued to occur for more than ten days after the drug was first given. Water consumption was markedly below normal, and the birds were reluctant to drink the apparently unpalatable medicated water.

It was seen that a variable amount of medicated water remained at the end of each day in lot H. Usually little or no water would be left in the drinking vessels of the other test groups. The amount of medicated water consumed daily could not be measured accurately since waste and evaporation were factors involved.

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Response of the Syrian Hamster to Rectal Instillation of Modified Newcastle Disease Virus

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THE ADAPTATION of the California strain 11914 of Newcastle disease virus (NDV) to the Syrian hamster with 300 intracerebral serial passages, with nine intranasal serial passages,[1] and with ten oral passages has been reported.[2] Brain material from the third oral passage, which originally was started from the 320th intracerebral passage and confirmed to be NDV by neutralization tests using hamsters injected intracerebrally as the test host, was used to initiate this experiment rectally in hamsters. No attempts were made to establish NDV in hamsters by rectal instillation previous to these studies.

The modified hamster virus was first successfully established by rectal instillation in 4 hamsters (ave. weight 30.0 Gm.) by inserting a small metal tube 1 in. into the rectum and instilling 0.1 cc. of a 10 per cent brain suspension of the third oral passage. The hamsters showed signs of involuntary motor reaction, excessive salivation due to pharyngeal paralysis, and paralysis in five to six days. These symptoms are similar to those occurring in hamsters infected by other routes.[3,4] Brains were removed aseptically from the moribund hamsters, ground with alundum, and diluted to a 10 per cent suspension with physiological saline. Of this suspension, 0.1 cc. was instilled rectally into hamsters. By using this technique, the rectal passages were carried through ten passages, as shown in table 1. These subinoculations are being continued at the present time.

To determine the distribution of Newcastle virus in the affected hamsters, suspensions of blood, liver, spleen, kidneys, lymph glands, lungs, spinal cord, feces, eyes, and brain from hamsters of the fifth and tenth rectal passages were injected into embryonating White Leghorn eggs. Because of possible bacterial contamination, each suspension was treated with streptomycin in the ratio of 1 mg. per cubic centimeter and with penicillin in the ratio of 500 units per cubic centimeter before egg inoculation. Of each suspension, 0.1 cc. was injected into the allantoic sac of each of 6, 9-day-old eggs. Virus was isolated from the brains and spinal cords of hamsters of the fifth and from the brains of hamsters of the tenth rectal passage. The virus isolated in these 3 cases was completely neutralized by positive Newcastle disease chicken serum but was not affected by normal chicken serum. Virus was not isolated in embryonating chicken eggs from the other suspensions. Neutralization tests were also made, using the brains and cords from hamsters of the fifth and the brains from hamsters of the tenth passage and using hamsters as the test host. The virus was again confirmed to be NDV.

TABLE 1—Rectal Passage of a Strain of Hamster-Adapted Newcastle Disease Virus

Passage (No.)	No. animals exposed	No. animals showing symptoms	Minimum-maximum incubation period (days)
1	4	3	5-6
2	10	4	4-6
3	6	6	3-5
4	12	10	4-5
5	15	14	3-6
6	8	8	3-5
7	12	5	4-6
8	6	6	3-5
9	6	6	3-6
10	6	6	3-5

SUMMARY

Hamsters were successfully infected by rectal instillation using virus which had been modified by 320 intracerebral passages and three oral passages in hamsters. Serial passage by rectal instillation was continued through ten subinoculations. Virus was isolated in embryonating eggs from the brains and spinal cords of hamsters of the fifth and brains of hamsters of the tenth rectal passage. The results of neutralization tests in embryonating chicken eggs

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and hamsters confirmed the virus to be that of Newcastle disease. Hamsters infected rectally showed symptoms similar to those in hamsters infected by other routes. These symptoms were involuntary motor reactions, excessive salivation from pharyngeal paralysis, general paralysis, prostration, and death.

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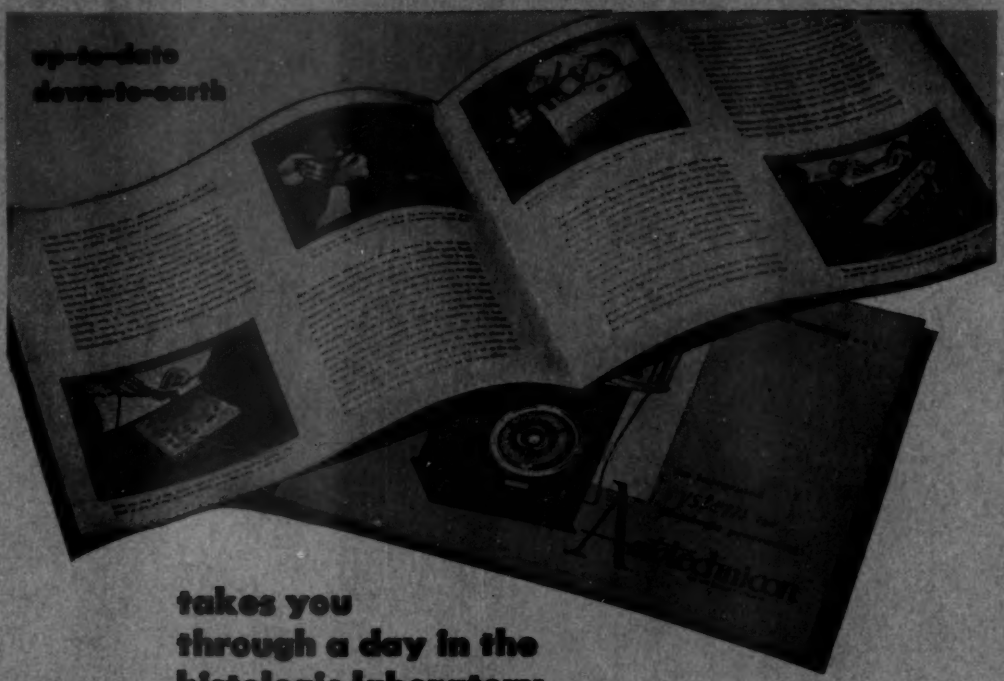

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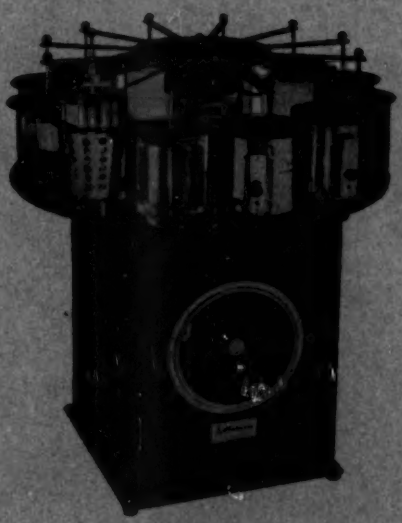

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